"The effect of biochemical changes upon certain biophysical phenomena of nerve activity: the functional significance of the connective tissue sheath of the peripheral nerve trunk".

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INTRODUCTION.

It has been known for a long time that the peripheral nerves in vertebrates consist of an aggregation of nervous tissue, the nervous elements, or nerve fibres, bound together by "indifferent" connective tissue. This binding tissue resembles connective tissue found in all parts of the body, and it might be supposed by analogy that its sole function is to support and hold together the nerve fibres, and that its presence in no way affects the behaviour of these fibres, so that for all intents and purposes it can be ignored altogether in all studies of peripheral nerve physiology.

The principal aim of the present work is to examine the possibility that this "indifferent" connective tissue of the peripheral nerves (the lamellated sheath, in particular,) plays a significant part in many physiological experiments because it is a barrier which prevents the diffusion of substances into or out of the nerve trunk.

It is hoped to show later that almost from the beginnings of electrophysiology many physiologists have been acutely aware of the nerve sheath and have realised that it might modify the apparent properties of nerves. However, in recent years, in the course of publishing the results of an extensive series of experiments performed with frog nerves,

Lorente de No has found it necessary to state categorically that the nerve sheath cannot be a diffusion barrier and cannot affect the apparent electrical properties of the nerve.

It may perhaps be asked whether this problem is really of such importance as to justify much labour to elucidate it. Lorente de No has said "if the epineurium were an effective barrier to the diffusion of solutes in general, and electrolytes in particular, and if it played an immediate role in determining the electrical characteristics of nerve, then all the work that has been done in the past with intact nerve trunks would stand in need of radical revision, because all the results obtained heretofore would have been vitiated by exceedingly important sources of error. Indeed, there would be in the literature on nerve physiology hardly a single important observation that could stand uncorrected". (1950, p.196). The case is a little overstated by him, and his statement would perhaps apply with even more truth to his own work. Yet it cannot be denied that the conclusions to be derived from studies of nerve behaviour point to totally different conceptions of nerve activity if the nerve sheath is assumed to be a diffusion barrier. The truth of this is very evident from the writings of Lorente de No, whose interpretations have led him into direct

opposition to the postulates of such outstanding hypotheses of nerve activity as those associated with the names of Bernstein and Hodgkin.

Bernstein's (1912) "concentration" hypothesis suggests that if the axon membrane is impermeable to anions and sodium ions the membrane potential may be a K potential which depends upon a difference in the concentration of K inside and outside the axon, and whose value should vary accordingly to $E = K \log \frac{C_1}{C_2}$. In Lorente de No's experiments (1947) however, there was no direct relationship between the demarcation potential and $\log \frac{C_1}{C_2}$ when nerves were placed in various concentrations of K. Accordingly he claimed that "these results cast very serious doubts upon the (Bernstein) theory" (1947, v.1, p.34).

Hodgkin's hypothesis (fully stated in 1951) is an attempt to interpret the nature of the action potential of the nerve fibre in terms of movements of Na and K through the axon membrane. It suggests that during activity there is an inward sodium current which depolarizes the membrane, and is responsible for the initial rising phase of the action potential. Hence it is crucial to the theory that a nerve should be incapable of conducting in the absence of external Na. But Lorente de No's experiments (1947) showed consistently that frog sciatic nerves can maintain conduction for many hours

in solutions of sucrose or choline chloride which contain no Na ions.

As a result, Lorente de No presented a number of hypotheses of his own (1947,1949,1950,1951) which take full account of his observations. He postulates thus that Na and K are important to the nerve only indirectly. They are only essential for the continued wellbeing of the nerves because they help to restore the primary substance on which all activity depends. They play the role of catalysts, perhaps, and so can only influence the state of the nerve when the reserves of these primary substances are exhausted. The hypothetical primary substances, he believes, may be quarternary ammonium compounds, because he found that certain such compounds may to a limited extent replace Na (1948, 1949). The resting potential, he states further, consists of a Q (quick) component, rapidly restored after depolarization, a L (labile) component, and a M component. These depend upon the presence of three electrical double layers, the E.M.F. being provided directly by oxidative metabolism. His extensive analyses of electrotonus in frog sciatic nerves under varying conditions (1947) have enabled him to correlate various components of the electrotonic deflections with hypothetical counter E.M.F's, produced at the Q, L and M double layers in the axon membrane.

It is only fair that Lorente de No's hypotheses should be considered objectively: but it is immediately evident that to a striking extent their integrity depends upon the assumption that the nerve sheath is not a diffusion barrier, and so does not affect either the rate of action of ions upon nerve activity or phenonema of electrical polarization in the nerve such as electrotonus.

The nerve sheath may have an important function in relation to the osmotic or metabolic requirements of the nerve fibres. Its properties are consequently well worth studying in themselves, as they may throw new light upon long term processes of nerve physiology.

It will be shown that there is some reason for considering the nerve sheath as only part of an extensive system of ectodermal sheaths which cover the entire nervous system, central and peripheral, and separate it from the surrounding mesodermal tissues. The general impermeability of these sheaths, together with selective permeability in certain specific regions, may serve to maintain the internal environment of the nervous system even more constant than that of the body as a whole.

One of the aims which guided the choice of methods in the series of experiments described in this thesis was to test the permeability of the sheath of frog sciatic nerves under such conditions

as will satisfy Lorente de No's criteria. Most of the previous observers in this field studied the effect of slitting or removing the sheath: Lorente de No, however, believes that any such procedure causes very important changes in the properties of the nerve fibres which may then be abnormally sensitive to Na lack for instance. He claims that "studies on the physiology of unaltered nerve fibres can only be carried out when the epineurium has been left intact on the nerve" (1950, p.231). This might seem to be an argument which, acting in a vicious circle, would preclude even the possibility of testing the original proposition. However, it is possible to introduce solutions into a frog nerve by its blood supply without in any way disturbing the nerve sheath. That is the basis of the method employed in the present experiments.

HISTORICAL REVIEW

The Connective Tissue of Frog Peripheral Nerves.

1. Histological evidence.

There has been little to supersede the careful but exhaustive investigations made in this field by 19th century observers, and in particular by Key and Retzius in Sweden, and by Ranvier in France. It would not be correct to say that physiologists and anatomists had previously been unaware of the presence of connective tissue in peripheral nerves, but ideas about its nature and exact distribution were very confused until Key and Retzius (1873,1876) and Ranvier (1875,1878) analysed its various components in great detail. As a result of this work, what had hitherto been known under the all-embracing but rather vague name of 'neurilema' became classified anatomically into a relatively simple system of connective tissue. This has facilitated considerably the description and the study of this tissue and the terminology suggested by Key and Retzius has thus become classical. That the latter should have become established, rather than that used by Ranvier is somewhat unfortunate, as the following considerations will show:

Key and Retzius	Ranvier
Epineurium	Tissu perifasciculaire
Perineurium	Gaine lamelleuse
Endoneurium	Tissu intrafasciculaire

The Swedish classification is no doubt euphonious and apparently very simple, but it has actually led to a great deal of confusion; the French one is fully descriptive and therefore quite unambiguous. The most striking and also the most important feature of the nerve connective tissue is the lamellated sheath which binds the nerve fibres into bundles. Practically all studies concerning the connective tissue sheath of the peripheral nerve have in fact dealt with the lamellated sheath. Its correct equivalent is the perineurium, yet many authors refer to it as the epineurium, i.e. the comparatively loose tissue which forms the outermost sheath, especially where there are several nerve bundles to be held together, (e.g.Lorente de No, 1950; Rashbass and Rushton, 1949; Lundberg, 1951 a). The frog sciatic nerve, which has been the subject of most investigations consists, of course, of only one bundle and has relatively little epineurium.

The descriptions by Key and Retzius, and by Ranvier agree fully in nearly every respect:

1) The perineurium or lamellated sheath has a structure of concentric lamellae, the number of these varying with different nerves and different animals: seven or eight is fairly typical for mammalian nerves, but it is not clear whether frog nerves have a similar number. Each lamella consists of a fibrillar network, covered on both sides by nucleated, endothelial cells. In frogs, the lamellae are more

homogeneous and less streaked than in mammals. The potential interlamellar spaces form a continuous serous cavity, which apparently communicates with corresponding endoneural spaces. There may be some connection with similar interlamellar epineural spaces, but according to Key and Retzius, there is no anastomosis with external lymphatic channels, and lymphatic drainage, if any, takes place within a closed system which includes endo- and perineural spaces and the subarachnoid space of the central nervous system. Ranvier, on the other hand, believed that drainage occurs outwards through the peri- and epineurium, but he gives little evidence to support his view.

2) The epineurium (or perifascicular tissue) has an inner component which invests the individual nerve bundles, and which has rather coarser lamellae and rather more fibrillar tissue than the subjacent perineurium. The more superficial layers are looser and much less regular in structure. Many fat cells, connective and elastic fibres, and blood vessels are found in it.

3) The endoneurium (intrafascicular tissue) may be subdivided into:-

a) a coarser lamellated fraction which takes the form of partitions running between groups of nerve fibres. It is continuous with the perineurium, and blood vessels penetrate into the nerve within its layers.

b) A rich fibrillar network, the fibrillenscheide, which has an incomplete cellular covering, and clothes and separates the individual nerve fibres. This layer covers the capillaries but numerous gaps sometimes allow capillaries to come into direct contact with the neurilema on the surface of individual axons.

The only studies that have added much to these 19th century descriptions are those of Plenk (1927), Laidlaw (1930) and de Renyi (1932). By a refinement of histological technique, Plenk and Laidlaw were able to describe in mammalian nerves an extremely delicate web of connective tissue fibrils lying on the axon outside the neurilema and forming the innermost portion of the endoneurium. Unlike the predominantly longitudinally disposed and coarser fibres of the fibrillenscheide the fibrils cannot be shown except by silver impregnation. De Renyi used a beautiful microdissection technique to show the intimate relationship between the endoneurium, the neurilema and the nerve fibre under comparatively normal conditions. The only histological studies of intact and desheathed nerves are those published by Causey and Palmer (1953). These authors came to the conclusion that the component which is removed in the process of desheathing frog and rabbit nerves is the epineurium, and that it is therefore the epineurium which is a diffusion barrier.

2. Physiological evidence.

a) The diffusion of substances into and out of the nerve.

Apparently the first to remark on the unexpectedly slow action of many substances on nerves is Eckard (1851) who was struck by the slow rate at which salts of Fe and Cu (which were known to coagulate proteins) block conduction in frog nerves. He thought that this was probably due to the connective tissue of the nerves. He noticed also that nerves placed in pure water or almond oil maintain conduction for a relatively long time. In contrast, substances which mix with fats, such as various volatile oils, carbon disulphide, etc cause a very rapid loss of conduction (in 8 - 10 minutes). A little later Kühne (1859) contrasted the quick stimulation of muscles with the slow stimulation of nerves in frogs by such chemical agents as various mineral acids and strong solutions of NaCl, KCl, $CaCl_2$. On the basis of such evidence, Hermann was able to say confidently in his textbook (1867) that the nerve substance (and the sheath, in particular,) allows only slow diffusion. Hence chemical stimulants to act on nerves must be in a more concentrated solution than is required in the case of muscles. In 1879, he stated even more strongly that without doubt, the sheath opposes a considerable resistance to the diffusion of substances into the nerve.

In the course of their investigations on the morphology of the connective tissue of peripheral

nerves, Key and Retzius, (1873, 1876) and Ranvier (1878) studied the distribution of coloured solutions injected into the various compartments of nerves. Such solutions as prussian blue in gelatin easily pass up and down the nerve when the tip of the canula is placed within a bundle or within the perineurium. They travel usually within perineural spaces, separating individual lamellae, and also within endoneural spaces, where they come into contact with the neurilema. According to Key and Retzius, the solutions spread in this manner to the serous cavities of the central nervous system but they never pass outward to the general lymphatic channels. The epineural spaces may also be filled to some extent, but extravasation only occurs when the injection is made within the epineurium itself. Ranvier, however, thought that lymph drainage from the nerves took place probably through the sheath, largely because he found that injected fluid does sometime reach the surface. Mulder (1938) however, has shown that solutions of dyes injected into a rabbit nerve under a controlled, moderate pressure do not escape out through the perineurium.

The chemical stimulation of frog nerves was the subject of many investigations in the second half of the 19th century. In a large series of experiments Grützner (1893) observed the effects of many electrolytes in strong concentrations (salts of

Na, K, Rb, Cs, Ba, St and Ca). He showed that chemical stimulation is much more effective at the central than at the peripheral end of the nerve, and he claimed that this can be correlated with the smaller proportion of connective tissue to be found centrally. Already in an earlier paper (1878) he had noticed a difference in the susceptibility of the motor and sensory roots of the frog sciatic nerves to block at high temperatures, and he had attributed it to a similar difference in their connective tissue content. Groves (1893) found that the central portion of frog nerves is less easily stimulated but they are killed sooner by strong NaCl solutions.

By the beginning of the 20th century, chemical stimulation had lost some of its interest. When Overton (1902 a,b;1904) undertook his exhaustive analysis of muscle and nerve physiology, he concentrated on : 1) the osmotic properties of these tissues, 2) the effect of Na lack, and 3) the action of various ions in isotonic solutions. He was the first to demonstrate convincingly that Na is essential for nerve and muscle function (1902 b). He also contrasted the rapid onset of inexcitability in muscles bathed in Na free sucrose solutions (within 45 minutes), with the 12 hours or so during which conduction is maintained by nerves placed in such solutions. In the last of these three papers (1904), he discussed at some length (pp. 251 - 260)

the effect of the connective tissue sheaths upon the rate of penetration of substances into nerves. Ranvier's description of the nerve sheaths, led Overton to believe that nerves are really just as sensitive to various substances as muscle but are shielded by their connective tissue. He then showed in actual experiments that the effect produced by various salts (e.g. K, Ca, Ba, etc.,) on nerves is, for any one concentration, very nearly the same as that produced on muscle if there has been enough time for the internal and external solutions to become equilibrated. He stated further that lipoid soluble substances (e.g. ether and alchohol) which easily penetrate into the protoplasm, pass rapidly through the endothelium of the sheaths, and also through the myelin sheath of the axon.

Some evidence that the electrolytes of a mammalian nerve diffuse out only very slowly is given by MacDonald's measurements (1900,1902) of the resistance of nerves at intervals after placing them into distilled water. Similarly Netter (1926) measured the specific resistance to direct current of frog nerves soaked in sucrose solutions. There was a relatively rapid change during the first few hours as the extracellular electrolyte diffused out, but he concluded that solutions to be really effective must be applied to nerves for at least 2 - 3 hours.

The first experiment to show clearly that removal of the nerve sheath may accelerate the penetration of a substance into a nerve was described by Rice and Davis (1928). According to these authors, chloral hydrate acts much more rapidly upon bullfrog sciatic nerves deprived of their outer sheaths. Feng and Gerard (1930) then showed that even slitting the external sheath has a very appreciable effect upon the rate of onset of conduction block, and the rate of recovery in normal Ringer, when nerves are tested with isotonic glucose, KCl and $CaCl_2$ solutions. The diffusion of methylene blue is also facilitated considerably by this procedure, and it should be noticed that fresh O_2 free Ringer only reverses asphyxial block after the sheath is slit. And so Gerard (1932), in a full review of the literature on nerve metabolism stated categorically that the connective tissue sheath is a diffusion barrier, and reviewed some of the evidence.

Meanwhile Bishop (1932) commented upon the much quicker action of KCl upon the frog spinal roots than upon the nerve trunk, which he explained by an easier penetration of the relatively unsubstantial sheath that is known to cover the roots (see also Erlanger, Bishop and Gasser, 1926,). Schmitz and Schaeffer (1933 a) anticipated to some extent the later work of Kato when they dissected from bullfrog

nerves a strand containing only some 100 fibres. They found that isotonic solutions of KCl and $CaCl_2$ produce a reversible block of conduction in such a strand within 10 - 20 seonds.

The technique of dissecting single fibres from frog nerves which was evolved by Kato enabled him and his associates to repeat many of the classical nerve experiments upon such preparations. Kato (1936) described the remarkably quick action of urethane, cocaine and Na free solutions upon single fibres, in which conduction was often blocked within 1 second. The action of Na free isotonic sugar solutions upon spinal roots was also found to be very rapid by Erlanger and Blair (1938). Conduction could be stopped within 10 sec. - 6 min., according to the thickness of the root and the proximity of a node of Ranvier.

It can be seen that up to the end of the 1930's for nearly a hundred years there had been a steady stream of evidence published from diverse laboratories that the connective tissue sheath impedes the free movement of substances into and out of nerves. For once the agreement among physiologists was universal. However in spite of the general unanimity, at the very outset of his "Study in Nerve Physiology" (1947) Lorente de No asserted "it is utterly impossible to believe that the connective tissue sheath of frog or bullfrog nerve could act

as a diffusion barrier" (v.1,p.23). He quoted experiments in which the effects of isotonic solutions of KCl could be detected within 1-2 minutes by means of cathodal polarization, and he referred to the dramatic action of NaCl on fibres deprived of Na: after a nerve has become inexcitable in a choline chloride solution, the addition of 0.015M Na is sufficient to cause some recovery in 1 minute. Lorente de No was very impressed by the independence of the nerve with regard to the external concentrations of K and Na, and he believed that new theories were necessary to deal with this (as mentioned earlier). He did a great deal of work (1949 a,b) to find substitutes for Na which might give a clue to the nature of the substances essential for nerve activity, in the production of which Na played an important but secondary role. He succeeded only partially, since he discovered that certain quarternary ammonium derivatives (which contain more than one ethyl group in the molecule) do restore the activity of some fibres in the absence of Na ions, but these were never A fibres, and the "restored" fibre had conduction velocities and action potentials which were very abnormal, (see also Crescitelli,1952).

The first reaction to Lorente de No's claims came in two papers published by Feng and Liu in 1949. In the first of these (1949 a) they compared the rate of onset of inexcitability and of depolarization and repolarization produced in intact and desheathed

frog and toad nerves by various chemical agents. They found that isotonic KCl, RbCl, $BaCl_2$, $CaCl_2$, glucose and choline chloride solutions, and solutions of cocaine and veratrine all act very much more rapidly on desheathed than on intact nerves. This difference was not found in the case of :-

(1) strong solutions of ethyl alchohol and acetone, which always produced a conduction block very rapidly.

(2) Solutions of enzyme inhibitors, such NaCN and iodoacetic acid derivatives, which had to act for several hours to produce inexcitabitity. Presumably in (1) the solutions diffuse through the sheath rapidly whereas in (2) the "action time" is so much greater than the "entrance time" as to make little difference to the total. Feng and Liu claimed, and gave some evidence, that the nerve is not injured by the removal of the sheath, but they did mention that sheathless nerves deteriorate more rapidly than intact nerves. Several coats of gelatin on the desheathed nerve did not, in effect, reproduce the sheath. In the second paper (1949 b) they showed that after the removal of the sheath, the resting potential of the nerve is, directly related to the log of the external K concentration, confirming the Bernstein theory. Rubidium gave similar results, but ether and veratrine did not.

More evidence followed. Keynes and Stämpfli (1949, cited by Stämpfli, 1952) measured the rate of

loss of ^{42}K from a frog nerve sheath which had been isolated and filled with a radioactive solution. Diffusion was apparently very slow until the sheath was washed with a solution of chloroform, when it became very rapid. Huxley and Stämpfli (1949) like Kato, found that single frog nerve fibres can be rendered inexcitable within 1 second by immersion in an isotonic Na free sugar solution, and Mullins (1950) showed that removal of the sheath increases the rate of uptake of radioactive phosphate by frog nerves to slightly more than twice the normal value. (Causey and Palmer (1953) have reported that the uptake of ^{32}P by rabbit nerves is increased 14 times by previous desheathing.)

The new contradictory evidence forced Lorente de No to re-examine the position; he performed another group of experiments to test the validity of his assertions, and published the results in 1950. He now claimed that he had fully confirmed his original statement, and made the following points:

(1). Removal or even slitting of the sheath or dissection of a single fibre is highly injurious to the nerve, whose properties are thereby altered fundamentally: e.g. it is sensitized to the action of Na free solutions.

(2). 0.5% cocaine causes a significant block of A fibres in a frog nerve within 1 minute, (total block in 40-50 minutes) hence the entrance is rapid, but the

action is slow.

(3). A nerve sensitized by lack of Na ions shows the effect of cocaine within 5 seconds (apparently there is an initial, temporary restoration of activity). Interference with the sheath which also potentiates the action of cocaine does so by sensitizing the nerve fibres.

(4). Normal Ringer restores conduction to fibres which have just become inexcitable because of Na lack within 2 seconds. The diffusion constant of Na can be estimated (Hill, 1928) since the following are known:

(a) the thickness of the sheath,

b) the external concentration of Na ions,

c) nerve fibres cannot conduct impulses unless the Na content of the surrounding fluid $\geq$0.010 M.

d) Hence the internal concentration of Na must have increased from 0 to 1/10 external within 2 seconds.

The value obtained, 1.2×10^{-4} cm^2 per minute, is about 1/10 of that for diffusion in pure water. Similarly, the coefficient of cocaine is about 1×10^{-4} cm^2 per minute.

(5). The rate of loss of excitability does not depend upon rates of diffusion but upon the previous state of the nerves:

a) A nerve made inexcitable by lack of Na (for 8-10 hours) and then allowed to recover in Ringer

for 20 minutes becomes inexcitable in a much shorter time than usual (e.g. in 30 minutes) in a Na free solution.

b) If recovery in the presence of Na ions is allowed to proceed for a longer time, then it also takes longer to block conduction again by lack of Na.

(6) There is an increasing gradient of sensitivity to lack of Na from the nerve trunks to the spinal roots.

(7) Most of the connective tissue of nerves is to be found within the nerve itself: the epineurium is only a small portion of the total. (A photograph is shown of a transverse section of the post ganglionic trunk of a cat's superior cervical ganglion, stained by Bodian's Silver Method.)

New observations on the effect of the epineurium on diffusion were given in another paper by Feng and Liu (1950) in which they described a method of "resheathing" desheathed frog and toad nerves. The rates of onset of inexcitability of resheathed nerves in KCl, cocaine and glucose solutions were intermediate in value when compared with the corresponding rates seen with intact and desheathed nerves. In spite of the unavoidable damage suffered by the sheath during removal it still acts as a substantial barrier when replaced.

Feng and Liu contrasted the rate of action of lack of Na or K on large spinal roots and on the

slender peripheral branches (which have a substantial sheath). They found that the spinal roots also give a linear relationship between the log of the external K concentration and the resting potential. Desheathed nerves were not significantly damaged because

i) their O_2 consumption was practically the same as that of intact nerves.

ii) In chronic experiments, nerves desheathed in situ, were removed after 2-3 weeks and their depolarization curves found identical with those of intact nerves. Feng, Hsu and Liu (1950 b) also showed by direct measurement that the diffusion of K into the nerve is increased considerably when the sheath is stripped.

Lundberg, who had worked with Lorente de No did not agree with him on the subject of the nerve sheath. He showed (1951c) (confirming the results of Feng and Liu) that whereas a desheathed frog nerve may stop conducting within 3-4 minutes (in isotonic diethanol-dimethyl ammonium chloride), after replacing the sheath the same nerve will conduct for up to 4-7 hours in the same solution. He also showed that a strong K solution acts much more rapidly on the sciatic nerve when injected into the aorta of the frog than when applied directly on the nerve. In another paper (1951 a) he noted that the spinal roots are more sensitive than the trunk to a variety of depolarizing agents.

Further evidence along these lines was published by Crescitelli and Geissmann (1951) and Crescitelli (1951), who compared the blocking actions of antihistamine and other compounds (including amidone, benadryl HCl, hystadyl HCl, thephorin phosphate) on intact, desheathed, resheathed and "unsheathed" nerves - the sheath was removed, replaced and removed again - conduction block occured much more slowly in the case of the intact or resheathed nerves.

Using a different approach to the problem, Huxley and Stämpfli (1951) measured the resting and action potentials of frog single nerve fibres by a potentiometric method and found that the effects of Na lack and of K were practically instantaneous. The log of the external K concentration and the resting potentials were proportional over a large range. Hodgkin (1951) in the course of a review of the evidence for the lack of permeability of the nerve sheath, quoted some unpublished experiments by Keynes in which the penetration of ^{24}Na into nerves was accelerated by slitting or removing the sheath.

There were further publications from Lorente de No and his associate Gallego based to a large extent on a reiteration of the arguments presented in the earlier papers. Gallego (1951) compared the action of Na free solutions on normal and

degenerated nerves, and stated that the removal of the nerve sheath sensitizes the nerve to Na lack much more than prolonged Wallerian degeneration. Lorente de No (1951 a) investigated in even greater detail the different rates of blocking (and restoration) of conduction obtained according to the previous history of the nerve (i.e. varying the periods of soaking in weaker or stronger NaCl solutions). He also measured the resistance of the nerve by applying rectangular pulses of current and measuring the potential drop between two intermediate electrodes. When the ionic concentration of the external solution was altered, there was a rapid change in the resistance of the nerve which was over in about 4 minutes, and was followed by only imperceptible changes. Assuming that 90% saturation had been reached in 4 minutes, Lorente de No calculated a diffusion constant of about $1 \times 10^{-4} cm^2/min.$ and concluded that therefore the sheath cannot be a diffusion barrier. He asserted that Na probably has four important functions in nerves:

1). Regulation of the water balance.

2). An action on the resting potential (in sucrose solutions the demarcation potential is raised).

3). An action related to excitability.

4). Maintenance of the various fractions of the membrane potential (because Na lack quickly

affects the components of the membrane electrotonic potentials).

The lack of external Na gradually leads to the loss of intracellular Na which is apparently essential for (3) and (4) albeit indirectly.

In his next paper (1951 b), Lorente de No examined more closely the different properties of the trunk and the spinal roots. The roots were found to be more sensitive to the lack of Co_2; but he claimed that the distribution of high sensitivity to many agents extends beyond the duro-epineural margin into the trunk for some 10-12 mm. He noted also that after inexcitability has become complete for both root and trunk the last trunk fibres to become inexcitable often recover sooner than the spinal roots. The explanation given by Lorente de No for these findings is that at the roots there is a gradual transition from the central nervous system the fibres of which have very different properties from those found peripherally.

In the third paper (1951 c) Lorente de No gave further details about experiments concerning cocaine and Na deficient nerves, and calculated rather arbitrarily high diffusion constants for cocaine mainly on the strength of the rapid temporary recovery produced by cocaine under certain conditions in Na deficient A fibres. The last paper of the series, (Gallego and Lorente de No, 1951) described

the rapid restoring action of small increments of LiCl upon nerves deprived of Na, (an action known since Overton, 1902 b). It may become evident within 5 seconds.

More evidence that desheathed nerves rapidly become inexcitable in Na free solutions was given by Crescitelli (1952 a,b.) Isotonic sucrose, choline chloride or tetramethyl ammonium bromide can stop conduction within 2 - 15 minutes. It is interesting that Crescitelli was unable to restore conduction in Na deficient nerves with a tetraethyl ammonium compound.

In defence of his position, Lorente de No (1952 a) adduced some histological evidence that removal of the epineurium is injurious to the nerve. He showed that within 10 minutes of desheathing there is much swelling of the myelin sheaths and there are some changes in the axoplasm. In a personal communication, Lorente de No (1952 b) stated that the perfusion of frog arteries with choline chloride and other inert quarternary ammonium compounds in a gelatin solution had no effect on the sciatic nerve action potential even after 90 minutes. In contrast to this the outward diffusion of procaine from a nerve was found to be exceedingly rapid.

In another personal communication (1952 c), Lorente de No described experiments with an improved technique in which frog nerves perfused with Na free

solutions became inexcitable in as little as 24 min. He commented on the spontaneous recovery often seen if the perfusion is stopped, and also on the apparently greater sensitivity of the perfused frog muscles to Na lack. However, he stressed the difference between perfused and desheathed nerves (which are blocked in an average of 5 minutes according to Lorente de No) but expressed his belief that the mechanism of block as a result of Na deficiency is partly different in intact, perfused and desheathed nerves.

One should mention at this point a series of experiments concerning the curious phenomenon of partial recovery produced in asphyxiated frog nerves by washing with O_2 free Ringer under certain conditions (for earlier references see Gerard, 1932). Feng and Gerard, (1932) found that the recovery is only seen when the nerve sheath is slit. Cohen and Gerard (1933) thought that only some oxydising agents (e.g. $NaNO_2$) could produce the effect, although after a certain time most of them (including $NaNO_2$) had a depressant action on the nerve, even in the presence of O_2. Aykut and Winterstein (1950 a), however, showed with intact nerves that plain Ringer is at least as effective as $NaNO_2$ in inducing anoxic recovery. They described another surprising finding (1950 b); a frog nerve may recover from prolonged asphyxia only

if the Ringer in which it is being asphyxiated is not changed at intervals. If the Ringer is changed then the nerve takes rather longer to become totally inexcitable, but the block is no longer reversible in O_2. Aykut repeated these experiments with intact and desheathed nerves and also with single fibres (1952). He found that the same holds in all three cases, but the irreversible block is more rapid with desheathed nerve and single fibres. The addition of a little cat serum to the changed Ringer prevents the irreversible loss of excitability. From the above it seems possible that two processes are involved, at least one of which is affected by the presence of the nerve sheath:

1) The sheath prevents the outward diffusion of an injurious substance which accumulates in the interstitial fluid as a result of the anoxia, and this causes inexcitability. A recent paper by Feng, Hsu and Liu (1950 a) gave evidence that the substance in question may be K.

2) There is normally another substance present within the nerve which enables it to reverse the process described in (1) when O_2 becomes available. That substance, (present in cat serum), is lost only when the nerve is repeatedly washed by changing the Ringer. Aykut also mentions that $NaNO_2$ is only toxic to nerves which have been desheathed.

b) The electrical properties of the nerve connective tissue

The earliest reference to the high electrical resistance of the frog sciatic nerve sheath was found in a paper by Bunzen, published in 1807. Bunzen was the inventor of the "frog battery", a curious arrangement of nerves and muscles in series, such that the injury currents of the nerves were apparently made to stimulate the muscles. Because in his experience the external nerve sheath was not a good conductor, he recommended that it should be split and the nerve fibres brought out. He said that "it is indescribable how much the experiment is facilitated by this procedure". Harless (1846) expressed a similar opinion: he had found that whereas stimulation at a certain point of a frog nerve with a simple Voltaic cell gave no response, after removal of the "neurilema" similar stimulation at this same point was often effective. The "neurilema" he concluded is an insulator with regard to galvanic currents.

The insulating properties of the nerve sheath were considered in the first half of the 19th century mainly in relation to the theory that the impulse travels down the nerve as an electrical current (see Longet, 1842, and Du Bois-Reymond, 1849). Much of the evidence was confused and very unsubstantial. Du Bois-Reymond, as a result, dismissed what evidence

there was, and ignored the sheath in his analysis of electrotonus. Subsequently little attention was paid to the electrical properties of nerve connective tissue until the 1920's. One exception can be found in the measurements of mammalian nerve conductivity made by Woodworth (1903) who estimated the specific conductivities of sciatic and ulnar nerves; the latter was always greater, and he ascribed the difference to the smaller content of the ulnar nerves in connective tissue. He also found the specific conductivity of a strand freed from the sciatic nerve to be greater than that of the whole nerve which has a higher proportion of connective tissue.

The modern era began with a comprehensive study by Bishop, Erlanger and Gasser (1926) of the connective tissue sheath in frog nerves. They noticed that if the sheath is removed or the recordings are made from the spinal roots, the shock artefact is much diminished. They concluded that the nerve connective tissue is polarizable and that many of the properties of nerve as usually measured may be in fact the properties of this non-nervous structure. Ebbecke (1926) and Gildemeister (1928) did consider the distorting action of the connective tissue but they seemed to think of it more as a parallel ohmic resistance than as a polarizable structure. Bishop (1928 a,b) compared

the polarization curves obtained in nerves with those which might be expected on the basis of the cable theory, and showed that the connective tissue sheaths were probably responsible for the discrepancies. Spinal roots behaved very much more in accordance with the theoretical expectations.

In Germany Lullies' (1930) measurements of frog nerve polarization also suggested that the sheath is polarizable, and Schmitz and Schaefer (1933 b) found that a desheathed frog nerve dissected down to 1/6 of its original volume has practically no "polarization capacity" left.

Cole and Curtis (1936) claimed that removal of the sheath decreased the transverse resistance of a frog nerve to 1/5 of the initial value. Tasaki (1939) measured the chronaxie of frog nerves before and after removing the sheath. This procedure decreased the chronaxie to one half. In 1942, Tasaki described an experiment done together with Tsumematsu in which they showed that the phase of depression produced by subthreshold stimuli in intact nerves is not seen with desheathed nerves or single fibres. In another experiment, in collaboration with Takeuchi, the initial spike recorded when a direct current is applied to an intact nerve (cf. Lorente de No's fast electrotonus) did not appear when single fibres were used. Both these phenomena he attributed to polarization and

"action currents" of the cells of the connective tissue sheath. Further measurements of the resistance and the capacitative reactance of intact and desheathed frog sciatic nerves were made by Rössel (1943), who stated that the polarization of the nerves is reduced to 1/3 - 1/10 by removing the sheath.

As mentioned earlier, Lorente de No (1947) made an extensive analysis of the electronic potentials of frog sciatic nerves, attaching great physiological significance to the various components of the fast and slow electrotonus. He assumed that these are directly related to states of nerve activity, and in defiance of the general agreement that the nerve sheath is a polarizable structure, in no way dependant upon, or affected by, the presence of the sheath.

Rashbass and Rushton (1949) showed that several anomalies in nerve behaviour from the point of view of the simple cable theory disappeared to a large extent when the external sheath is removed: for instance, the excitability curve fits an expression of the type $e^{-\frac{x}{\lambda}}$ very much better, and excitation takes place much nearer the cathode. By passing an electrode within an intact nerve they also showed that there is a considerable potential difference across the sheath when current flows through the nerve. Further, the electronic potentials of a

desheathed nerve are much reduced from which they concluded that the interpretation of electrotonic potentials in intact nerves is a very difficult matter.

Lorente de No (1950) could not accept the conclusions reached by Rashbass and Rushton. He pointed out that the slow electrotonus is reversibly modified by many agents, e.g. CO_2, metabolic inhibitors, asphyxia and changes in temperature; hence, he claimed, it is evident that the slow electrotonus cannot be caused by the properties of the epineurium. On the other hand, he examined the fast electrotonus in three different nerves from the turtle in which there was no correlation between the thickness of the epineurium and the amplitude of the fast electrotonus. The fast electrotonus was rapidly abolished by ether but Lorente de No believed there was no damage to the "epineurium" because the three nerves behaved characteristically in this respect also.

In the course of an investigation of strength-duration curves for the excitation of frog nerve, Schoepfle and Susman (1950) found that "the perineural sheath so complicates the impedance and distribution of current as to obscure a basic relation between excitation, and local membrane voltage" Schmitt and Stewart (1950)(after studying the electrical admittance of the nerve membrane

by the complex attenuation method) claimed that "frog nerves have average membrane properties similar to those of spinal fibres, but these properties are almost completely obscured if the nerve is not freed of its external membranous sheath".

Lundberg (1951 c) repeated some of the experiments of Lorente de No, and Rashbass and Rushton, with desheathed nerves and with spinal roots. He found the fast electrotonus to be much smaller than in the intact nerve trunk. The decrement of the slow electrotonus was more truly exponential, and the characteristic length of the nerve (λ) was much less variable. These properties were reversed by resheathing a desheathed nerve. Using an internal electrode, he confirmed the presence of the large potential difference found by Rashbass and Rushton across the epineurium when a current is flowing. His final opinion was that the slow electrotonus is probably not produced by the sheath but that it is significantly distorted by its electrical properties.

Further experiments by Lorente de No quoted in 1952 (a) gave results which agree on the whole with those obtained by Lundberg. The isolated "epineurium" of a frog nerve only has a fast electrotonus; in situ, a small hole made in the "epineurium" reduces the fast electrotonus, but has no action on the slow electrotonus. However, the results also

suggested that the change in the fast electrotonus seen after desheathing is a gradual one and occurs only after a latent period of some minutes: Lorente de No's interpretation was that the effect is really secondary to osmotic changes within the nerve.

METHOD.

The artificial perfusion of the frog is an old established experimental technique. It has been used for a wide variety of purposes for over 50 years, but not for the specific study of peripheral nerve function.

The "whole frog" preparation was described by Verworn (1900) who inserted a canula into the aorta of the frog, and perfused the entire circulatory system with the solutions of strychnine whose actions he was investigating.

The "hind-leg" preparation is widely known as the Läwen-Trendelenburg preparation after the two German physiologists who are usually believed to be the first to describe it. However, several years before Läwen's paper, Cushing (1901) gave a full account of a typical frog hind leg perfusion for the study of the behaviour of muscles in the presence and absence of K and Ca. The canula was tied into the descending aorta from the dorsal aspect thus keeping the necessary incision as small as possible. The roots of the sciatic plexus were sometimes stimulated to distinguish between direct and indirect excitation of the leg muscles.

The only refinement of technique introduced by Läwen (1904) was to fix a canula into the anterior abdominal vein so as to obtain an accurate measure of the effect of suprarenal extracts on the peri-

pheral circulation from the rate of outflow. Trendelenburg (1910) tried to reproduce normal conditions more closely by having a pulsatile perfusion pressure; in his hands, the preparation became a method of assaying the adrenaline content of normal blood.

Frog perfusion methods have been used frequently since the pioneer experiments described, and no attempt will be made to review this field. However, a few examples will be mentioned to show some of the possibilities of this technique. Drinker (1927), Saslow (1938), Danielli (1940), and Hyman and Chambers (1943) all found it convenient for the study of oedema formation; while Mond and Anson (1928) were able to determine by its use the permeability of muscle cells to various ions. Rivkine (1950) demonstrated a relation between the circulating Ca and K levels and the E.E.G. in the perfused frog.

It is not, however, until the most recent years that the peripheral nerve trunk has been the principal object of investigation. That this should have happened in connection with the problem of the permeability of the nerve sheath is hardly surprising. The significance of the method as a means of bypassing the perineurium was recognised by Lundberg (1951) but the method was developed independently in the course of the present work.

Lorente de No, as mentioned earlier, described in personal communications (1952 b,c,) the results obtained when he perfused frogs with Na free solutions.

Frog Sciatic Nerve Blood Supply.

The gross vascular supply of the sciatic nerve in Rana Temporaria and Rana Esculenta was described very fully by Mies (1926). A large number of fine arteries pass to the nerve and to the cords of the lumbo-sacral plexus from the aorta, the common iliac and the sciatic arteries and their branches. The nutrient vessels are especially prominent where the nerve crosses a main artery (e.g. at the pelvic outlet, or near the sciatic bifurcation in the thigh). At the surface of the nerve trunk, the nutrient arteries divide regularly into central and peripheral branches, which anastomose extensively to form a continuous vascular network in the epineurium.

Vessels penetrate the perineurium, and reach the interior of the nerve usually within one of the thicker strands of lamellated endoneurium. The capillaries bear a close relationship to the individual nerve fibres from which they are usually separated by endoneural tissue but Ranvier (1878) claimed that they sometimes come into direct contact with the nerve fibres.

According to Mies, vascular anastomoses in the

frog are so rich that nothing short of ligation of the aorta at its origin will stop blood reaching the sciatic nerve.

Present perfusion method. (Fig. 1.)

All experiments were made with the common frog (Rana Temporaria) during a period which covered the four seasons; the preparations were, on the whole more satisfactory and easier to handle during the colder months. No discrimination was made between the sexes.

The frog's brain and spinal cord were pithed in every case. Läwen found that after pithing there was a great increase in the rate of flow, no doubt a result of the loss of the spinal vasomotor tone.

The metal canula was made from a hypodermic syringe needle: two sizes were used, a no.1 and a no. 19 needle, the larger one (no.19) only occasionally when an exceptionally large specimen was available.

The dissection can be either from behind or from the front. The latter is safer when dealing with a small frog in which the vessels are slender and the bifurcation of the descending aorta may be rather high. The dorsal approach is simple, and relatively quick; it is preferable when the frog is a large one.

The canula was usually inserted into the descending aorta, (in a few cases the insertion was made into the common iliac artery) with the help of magnifying glasses (x 2) and tied in position. One of the iliac arteries was ligated and the homolateral sciatic nerve with its peroneal branch dissected down to the level of the ankle and excised. This nerve was mounted in a nerve bath containing two sets of silver electrodes, so that the roots were stimulated and the action potentials recorded from the peroneal nerve. The bath had a central compartment of a capacity of about 7 cc. in which the middle 3 cm. of the nerve lay. It could be filled with solutions, and emptied, within 5 - 10 seconds.

The other leg and the pelvis, with the attached canula, were severed from the trunk and weighed. They were then placed in a moist chamber which had electrodes arranged so as to stimulate the roots of the sciatic nerve and record the action potentials from the peroneal nerve below the knee. To avoid interference by muscle potentials, the gastrocnemius, peroneus and tibialis anterior were ligated and resected. The peroneal nerve was always crushed between the recording electrodes.

The canula, which was about 1.5 cm long, was directly connected to a 3-way tap which controlled

the choice of perfusate. The solutions were allowed to run into the hind limb from bottles suspended at a height that varied between 30 and 50 cm above the chamber, according to the rate of flow, to keep the pressure within the aorta between 20 - 30 cm H_2O. Figures for the frog's arterial blood pressure in the literature vary between 10 and 40 mm. Hg., with an average between 20 and 30 mm.Hg. (Burton-Opitz, 1920; Prosser et al, 1950). The resistance of the narrow canula was estimated empirically so that the pressure in the aorta could be found from curves relating flow rate to pressure drop for different viscosities.

A continuous record of the perfusion flow, which was mostly of the order of 1 - 1.5 c.c./min., was made by a conventional drop recorder situated under a hole in the floor of the chamber. The temperature of the perfusates and of the interior of the moist chamber was recorded at intervals during an experiment; the average varied with room temperature (18 - 22°C.) but the fluctuations seldom exceeded 2 - 3°C in any one experiment. The preparations usually survived and functioned well for many hours (12 - 24 hours or more).

Solutions.

It can be seen from fig. 1 , that the perfused nerve and the non-perfused control always had a

common source of supply as far as the standard and test solutions were concerned. The solutions were mostly made up with Dextran; in a number of experiments gelatin was tried, and in a further number of experiments no colloid of any kind was added. The colloid solutions were employed to reproduce normal osmotic conditions as nearly as possible; this was particularly important when perfusing with Na free solutions, to prevent filtration playing an important part in the rate of exchange and thus making an analysis of the rate of diffusion impossible.

The "standard" solution contained the usual amounts of electrolytes:

Na	110.0 mM.
K	2.65 mM.
Ca	1.1 mM.
Cl	110.0 mM.
Bicarbonate	2.5 mM.
Dihydrogen phosphate	0.15 mM.

The buffers were salts of K. This ratio of bicarbonate and phosphate gives a pH of 7.6 - 7.7. In all the experiments, the pH was controlled by means of B.D.H. standard indicators; it was never allowed to pass out of the range 7.2 - 8.2, except when investigating the action of an acid solution, (the pH of frog plasma was given by White, 1924, as 7.65).

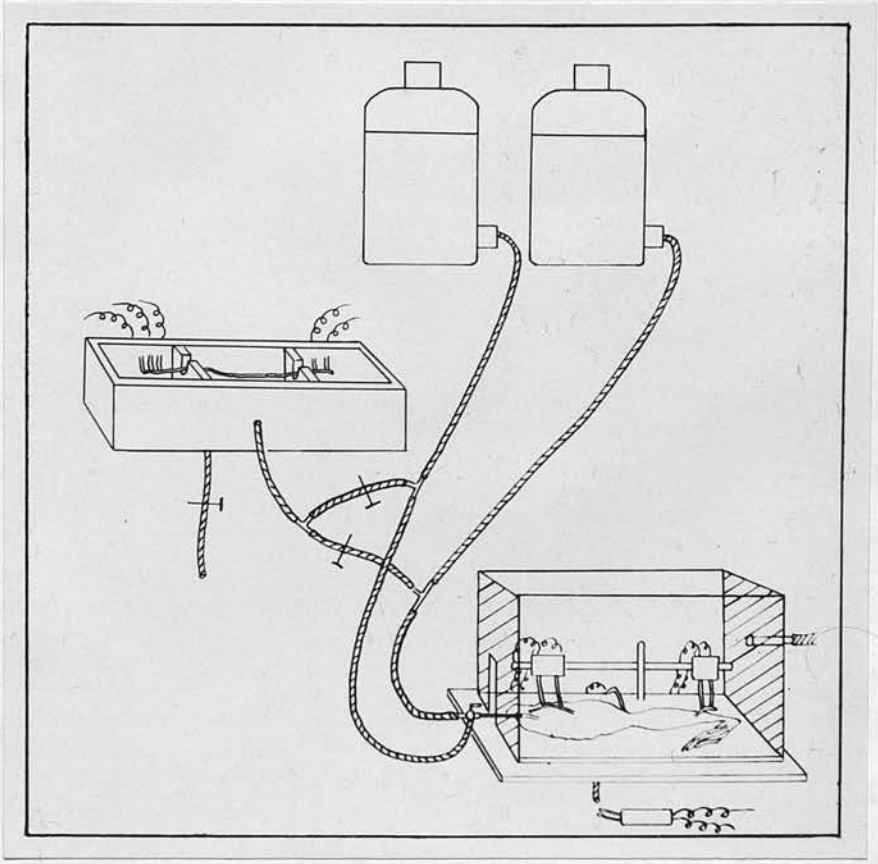

Fig. 1. Diagram showing a perfused frog hind limb in the moist chamber, and a non-perfused control nerve in the nerve bath.

The Na free solutions retained all the other standard components. The following have been used as osmotic substitutes for Na :

Sucrose	0.22M
Glucose	0.22M
Choline Chloride (Roche Products Ltd)	0.11M

When the effect of Na poor solutions was studied, the required concentration was obtained by mixing suitable proportions of Choline Chloride solution and standard solution.

The ordinary commercial Dextran contains 0.9% NaCl; the Dextran in the present experiments was a deionized fluid made available by Dextran Ltd. The Na content of this deionized Dextran and also of the commercial gelatin used were estimated after ashing by the Zinc Uranyl Acetate photometric method. The Dextran had a quite unappreciable amount of Na (less than 0.10 mM). The gelatin, however, had a sufficient Na content to give a final concentration of 0.35 mM in the 0.5% gelatin perfusate (or 0.70mM in the 1% gelatin). However, it can be shown that the diffusion time should not be increased by more than about 4% even with 0.70 mM.

Other test solutions were made up by displacing equivalent amounts of Na, but the concentration of the latter was usually not allowed to fall below 10% of the normal to avoid interference by the effects of Na deficiency.

Since Dextran is rather viscous (an Oswald viscometer gave values of about 4.5 for the specific viscosity), it was diluted with 50% its own volume of water: this brings the viscosity rather nearer the only published figure of frog blood viscosity, which is 2.4 (Burton-Opitz, 1902).

From the molecular weight of Dextran (given by the makers as in the region of 90,000), the colloid pressure of the diluted fluid (4%) should be about 10 cm of water. This can be compared with the following measurements of the frog plasma colloid pressure.

5.5 - 6.0 cm water (Krogh, 1922)

9.6 - 11.5 cm plasma (White, 1924)

7.1 cm water (Churchill et al., 1927)

11.5 cm water (Landis, 1927)

Gelatin perfusates were used at the suggestion of Lorente de No (personal communication, 1952 b). They were found less satisfactory than Dextran solutions because the viscosity increases rapidly, so that they tend to set at room temperature, and the pH was found more difficult to control over long periods. The concentrations tried were 0.5% (Chambers and Zweifach, 1940) and 1% (Chambers and Zweifach, 1944). The solutions were sometimes autoclaved to increase their fluidity. It will be seen later that the results obtained with 1% gelatin were appreciably slower than those obtained with either 0.5% gelatin or Dextran.

There was always some oedema after several hours of perfusion, shown by an increase in weight of the order of 30 - 50% (after 6 - 8 hours). The difficulty of preventing oedema altogether when perfusing with synthetic substitutes is well known. Different authors have claimed that the lack of various specific constituents of normal blood is responsible for an abnormal permeability of the capillaries: the red cells, (Krogh, 1922) the serum, (Drinker, 1927; Chambers and Zweifach, 1947) the red cells and O_2, (Saslow, 1938) the platelets (Danielli, 1940).

Recording and stimulating apparatus.

The electronic recording and stimulating apparatus was constructed for these experiments. It consists of a double channel, R.C. coupled, differential input amplifier and main amplifier, whose output is displayed on a double-beam oscilloscope, where it can be photographed by means of an aircraft camera adapted for the purpose.

The stimulator can provide either two independently variable stimuli (on one or two channels) triggered by the time base, or series of impulses initiated by a multivibrator, with a frequency range of 1/6 - 500/sec. The stimuli are condenser discharges whose shapes are somewhat distorted by the output valve and also by the transformer which feeds

them to the electrodes. Three time constants are available of which the medium one was used most, (duration of initial spike = 1 msec.). It should be assumed that stimulation was in all experiments maximal for A fibres (the only fibres studied) and at about 1 - 5/sec., unless it is stated otherwise in the text.

Timing signals (1 - 100,000 c/s) are supplied by a crystal controlled series of dividing circuits. All the photographic records are single time base sweeps. The procedure was simplified by the use of a Uniselector relay which in turn opened the camera shutter, triggered the time base and operated the camera motor, for single records, or for a series of records at intervals controlled by the rate at which a cathode follower recharged a condenser through a variable resistance.

Resistance measurements were made with a Wheatstone bridge circuit and a source of sinusoidal voltage at 1000 - 3000 c/s (at about 50 mV). The bridge was balanced with standard resistances and capacities, and the nul point detected with the aid of the amplifier. The accuracy was rather better than 1% up to 100,000 Ω, and about 5% up to 1M (over the higher range, the fixed arms ratio was raised from 1:1 to 10:1).

Histological methods.

The sciatic nerves examined microscopically

were mounted on lengths of stiff paper and fixed in either alchohol or Fleming's osmic-bichromate solution. The latter gave the most satisfying results, but absolute alchohol (after preliminary soaking in 60% alchohol) fixed much more quickly without any significant shrinkage. After dehydration and clearing, the nerve was imbedded in paraffin and sections cut as usual. The various stains employed included: Aurantia, Biebrich Scarlet, Haurob's, Isamine Blue, Mallory's, Picro Black and Ranvier's Picro Red.

Sections of nerves fixed in Fleming's solution had to be bleached in nascent Cl_2 or O_2 when further staining was wanted. A few nerves were treated with strong acetic acid which causes collagenous tissue to swell, and so stand out more clearly. Silver staining by Laidlaw's method required fixation in Zenker's fluid, and the paraffin sections were fixed to slides by Masson's gelatin glue, which was hardened in hot formol fumes. (Full details are given by Laidlaw, 1929; 1930). The method is long and laborious because its success depends upon a careful control of the duration of immersion and the temperature in the silver solution and this can only be achieved if one slide is taken through at a time. General precipitation of the silver occurs rather easily, especially if the slide is not washed well in distilled water, and inadequate volumes of 1% formol are used.

Other methods.

1. Demonstration of blood vessels.

a) The classical perfusion with soluble prussian blue in gelatin gave good results. The perfusion is at a temperature of about 37°C, and the nerve dissected out after the gelatin has set. The vessels are shown clearly, but the procedure is rather elaborate, and the raised temperature and relatively high viscosity of the fluid may affect the apparent distribution of the capillaries (although it should be added that there was little evidence to support this).

b) Indian ink, diluted and filtered through Whatman no.5 paper, was simple and quicker to use: it showed the blood vessels but did not outline them as clearly as gelatin.

The perfusion pressure was usually somewhat higher than that employed in the experiments because of the greater viscosity of the fluids; if necessary the vessels were cleared of blood by preliminary washing with Ringer until the escaping fluid lost its pink colour. The Indian ink or gelatin solution was then perfused for 10 - 15 minutes. The nerves were fixed in alchohol or Fleming's solution, dehydrated, cleared and then either mounted whole on the slide in damar, or imbedded in paraffin. The sections were often counterstained with Aurantia.

2. Intraneural injections.

A no. 18 hypodermic needle was inserted into the sciatic nerve most often centripetally at the bifurcation in the thigh; the insertion was also made in a centrifugal direction into the nerve at the junction of the two largest cords of the lumbo-sacralplexus in the pelvis. The needle could be seen through the perineurium and its position defined reasonably clearly when it was pushed some distance along the nerve. A ligature was sometimes placed around the nerve and the needle to prevent the escape of fluid but this was not really necessary when the needle was well in. Compressed air and a mercury manometer provided a fully controlled head of pressure: the flow rate was so slow (except at very high pressures) that it can be assumed there was little pressure drop along the needle. Straight injection with a syringe was also tried. The solutions injected were: 1. methylene blue, 1% and 0.1%; 2. 1% silver nitrate. To fix the methylene blue, the nerves were treated in saturated ammonium picrate and ammonium molybdate before the usual fixing and dehydrating procedure.

3. Permeability of the capillaries.

The whole frog, made insensible by urethane, was perfused with certain solutions which have been found suitable for testing the blood brain barrier

(Wislocki and King, 1936). They were: 1. aniline blue, 0.1%; 2. acid fuschin, 1.0% and 0.1%; 3. 1% mixture of iron ammonium citrate and K ferricyanide.

The prussian blue reaction was brought about by fixing the tissues in 10% formalin containing 2% HCl. The results were estimated by a simple examination of the various parts except in the last case where histological sections were made of the sciatic nerve, and of a muscle and a piece of gut for comparison.

4. Desheathed nerves.

Some effects of Na poor solutions were investigated with desheathed frog nerves. The desheathing method resembled that described by Rashbass and Rushton (1949). The perineural sheath was gradually freed at the main bifurcation of the nerve and with needles and fine watchmaker's forceps it was then peeled off the main trunk by rolling it upwards towards the roots. One of the two main branches was desheathed in a similar manner. The whole operation was performed with the aid of a binocular dissecting microscope. It was found that some experience was required to distinguish the rather delicate and transparent sheath of the frog nerve.

5. Phalangeal preparation.

The sciatic nerve was dissected from the

lumbo-sacral plexus to one of its peripheral digital branches, i.e. as far as the tip of the 3rd or 4th terminal phalanx, as described by Erlanger and Blair (1934). The phalangeal portion contains only some 2o - 30 nerve units.

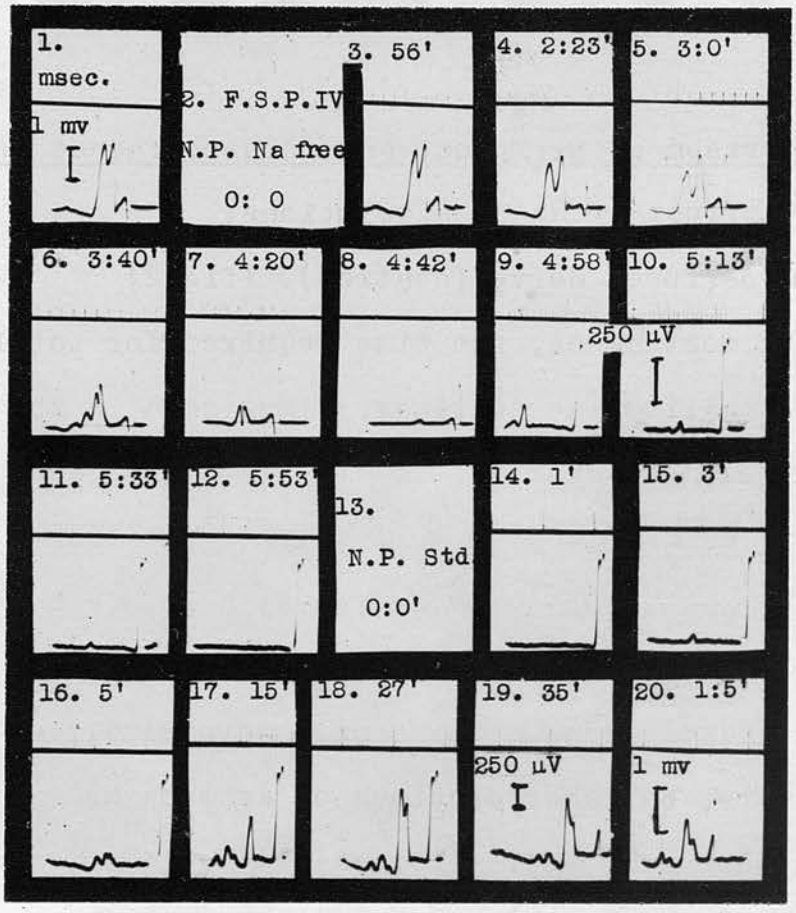

Fig. 2.A. Maximal action potential (with stimulus artefact) of a non-perfused frog sciatic nerve after various intervals in a Na free (0.11M Choline Chloride) solution, and then in the standard solution.

No.1 is a normal control in the standard solution at the beginning of the experiment.

Read each record from right to left.

The amplification was varied.

RESULTS.

I. Comparison of perfused, and "non-perfused" nerves:-

1. The effect of Na free solutions:

a) Non-perfused nerve (control). (fig.2)

In most cases, the time required for total inexcitability was distinctly less than in the experiments of Overton (1902; 12 hours) and Lorente de No (1949; 8 - 10 hours for A fibres, 14 - 16 hours for B and C fibres) probably because those authors worked with bullfrog nerves of greater dimensions. Six hours were usually necessary, but shorter times of as much as 3 - 4 hours were recorded. Lorente de No (1944) and Crescitelli (1952 b) have commented on the variable sensitivity of frog nerves to Na lack, for which there seems to be no simple explanation.

The importance of the size of the intact nerve in determining its ability to withstand Na lack was shown in a few experiments with a phalangeal preparation. Total block occurred in such a preparation within 40 - 45 minutes in a Na free solution. The smallest diameter of the nerve exposed to the solution was of the order of 150 μ . The rate of recovery in a Na containing solution depends upon the time during which the nerve is allowed to remain in the Na free solution after the inexcitability has occurred. If the solution is changed

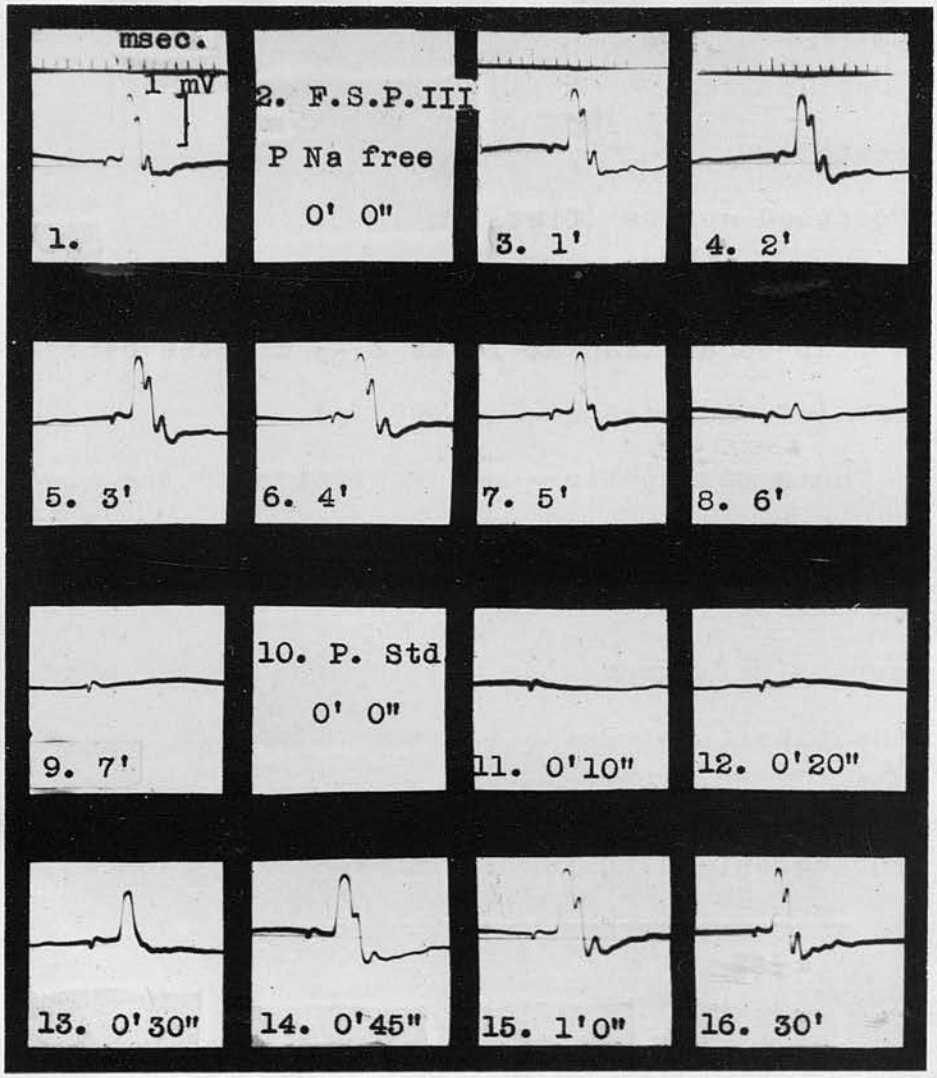

Fig. 3. B. Maximal action potential (with stimulus artefact) of a frog sciatic nerve at various intervals after beginning perfusion with a Na free (0.11M Choline Chloride) solution , and then with the standard solution.

No.1 is a normal control, perfused with the standard solution, at the beginning of the experiment.

Read each record from left to right.

There was some 50 cycle mains interference.

without appreciable delay, conduction returns within 1 minute.

b) Perfused nerves (fig.3).

It was always found that the muscles of the limb stop conducting at least 2 -3 minutes before the nerve. (cf. Lorente de No, 1952 c).

There was considerable variation in the blocking time and there ~~were~~ was a number of occasions on which the perfusion failed to produce a block after 2 hours, (cf. Lorente de No, 1952 c). The nature of the experiment makes it very difficult to control the perfusion of the nerve itself, but it was found by subsequent perfusion of the vascular system that negative results were usually associated with inadequate filling of the vessels, Sometimes, when the nerve vessels were exceptionally difficult to demonstrate, the cause of the failure was rather obscure. The flow rate in these cases was always very low, so that some defect of perfusion was present which prevented rapid exchange of fluid in the nerve.

The blocking time did not apparently vary with the Na substitute, but it was appreciably longer with 1% gelatin than with Dextran or 0.5% gelatin perfusates.

Table 1.

Perfusion with a Na free Dextran perfusate.

Na substitute	Blocking time (min)	Initial recovery time. (sec)
Sucrose	25	20
Sucrose	21	10
Sucrose	19	25
Glucose	19	10
Glucose	15	5
Choline Chloride	14	10
Sucrose	10½	10
Sucrose	6½	10
Choline Chloride	6½	20

Table 2.

Perfusion with Na free gelatin perfusates.

Gelatin concentration	Na substitute	Blocking time (min)	Initial recovery time. (sec)
1%	Choline Chloride	32	60
1%	Choline Chloride	32	25
1%	Glucose	24	10
0.5%	Choline Chloride	20	10
0.5%	Choline Chloride	7½	10

It can be seen that blocking times of the order of 7 minutes were obtained with sucrose and choline chloride in the case of Dextran perfusates, and with choline chloride in the case of a ½% gelatin perfusate. The time to initial recovery when perfusing with the standard solution did not vary

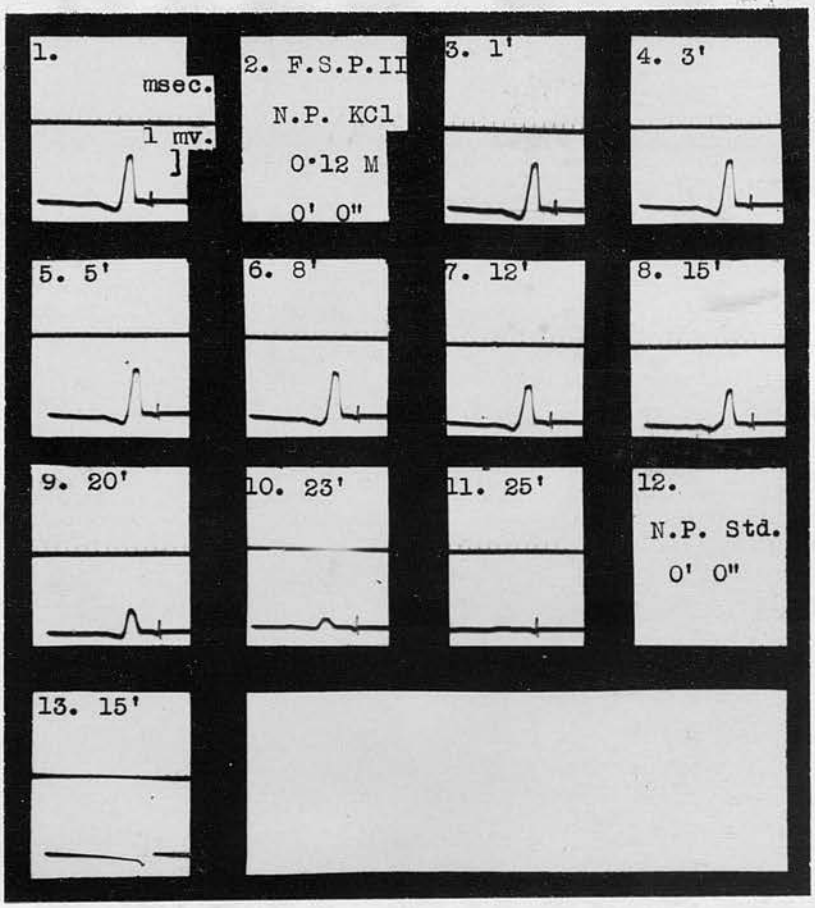

Fig. 4. A. Maximal action potential (with stimulus artefact) of a non-perfused frog sciatic nerve after various intervals in 0.12M KCl, and then in the standard solution.

No.1 is a normal control in the standard solution at the beginning of the experiment.

Read each record from right to left.

There was no recovery in this case.

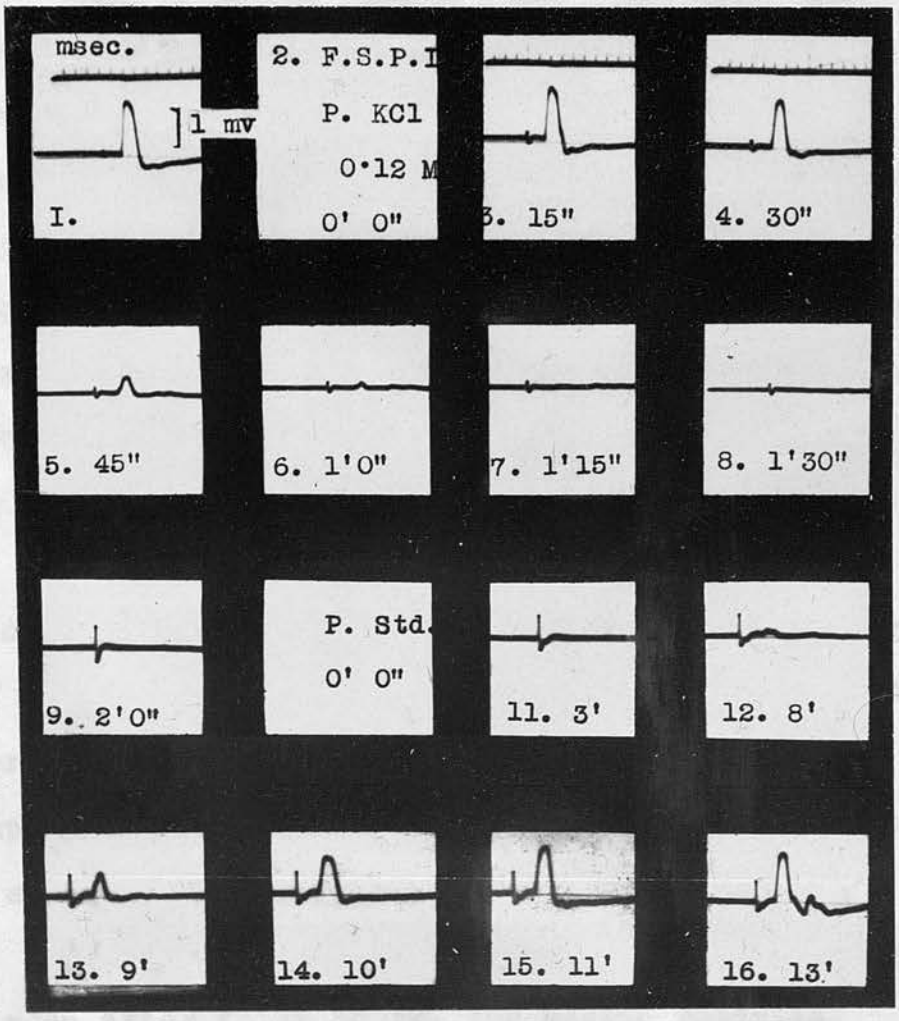

Fig. 5. B. Maximal action potential (with stimulus artefact) of a frog sciatic nerve at various intervals after beginning perfusion with 0.12M KCl, and then with the standard solution.

No.1 is a normal control, perfused with the standard solution, at the beginning of the experiment.

Read each record from left to right.

The stronger stimulus has caused a double response in No.16.

much, although it tended to be rather long with 1% gelatin, as might be expected. The complete recovery time is rather difficult to estimate exactly since there is no sharp change associated with full recovery. Therefore, no attempt was made to study this before investigations of the effects of small changes in Na concentration.

To show that the loss of excitability was not due to choline chloride, a nerve which had ceased conducting was perfused with a solution containing the usual amount of choline chloride and 0.05M NaCl. There was a rapid recovery of conduction, and the compound action potential regained its original form, in spite of the hypertonicity of the perfusate.

If the Na free perfusion was interupted before or soon after total block, the action potential tended to recover spontaneously to some degree, (cf. Lorente de No, 1952 c).

2. The effect of concentrated solutions of electrolytes.

i) 0.12M KCl.

a) Non-perfused nerve. (fig.4)

The action potential began decreasing after about 8 minutes and disappeared altogether in about 25 minutes. Recovery in the standard solution was slow; in two cases it began after some 20 minutes, but in a third case the block was irreversible.

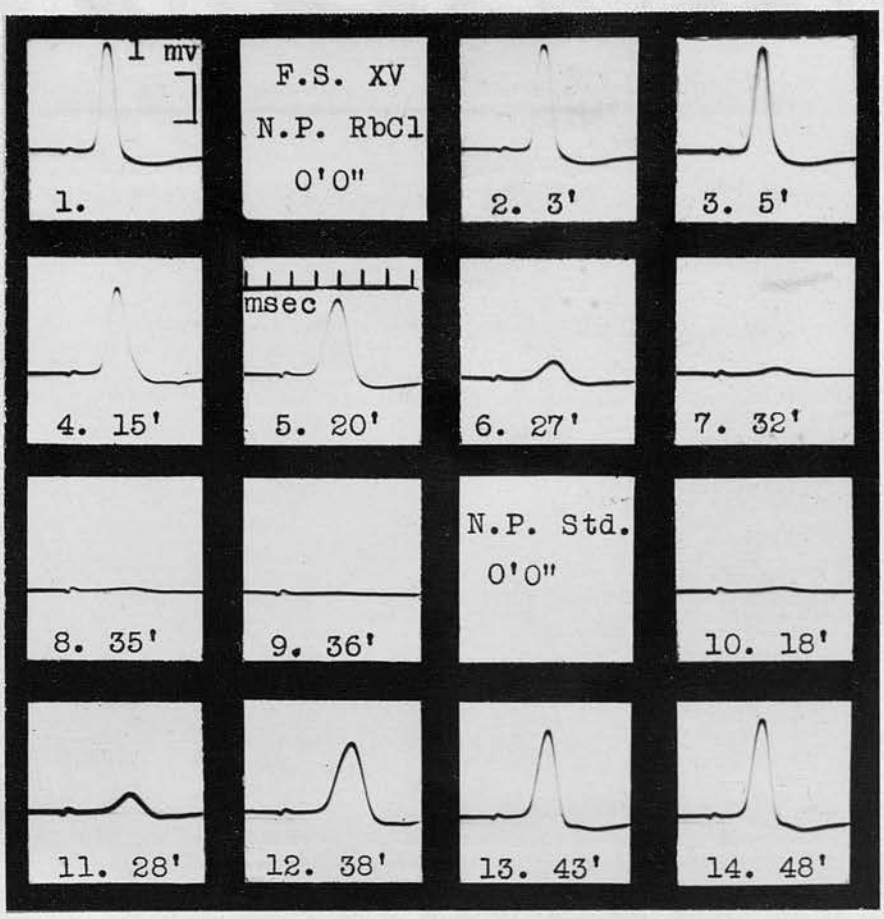

Fig. 6. A. Maximal action potential (with stimulus artefact) of a non-perfused frog sciatic nerve after various intervals in 0.10M RbCl, and then in the standard solution.

No.1 is a normal control in the standard solution at the beginning of the experiment.

Read each record from left to right.

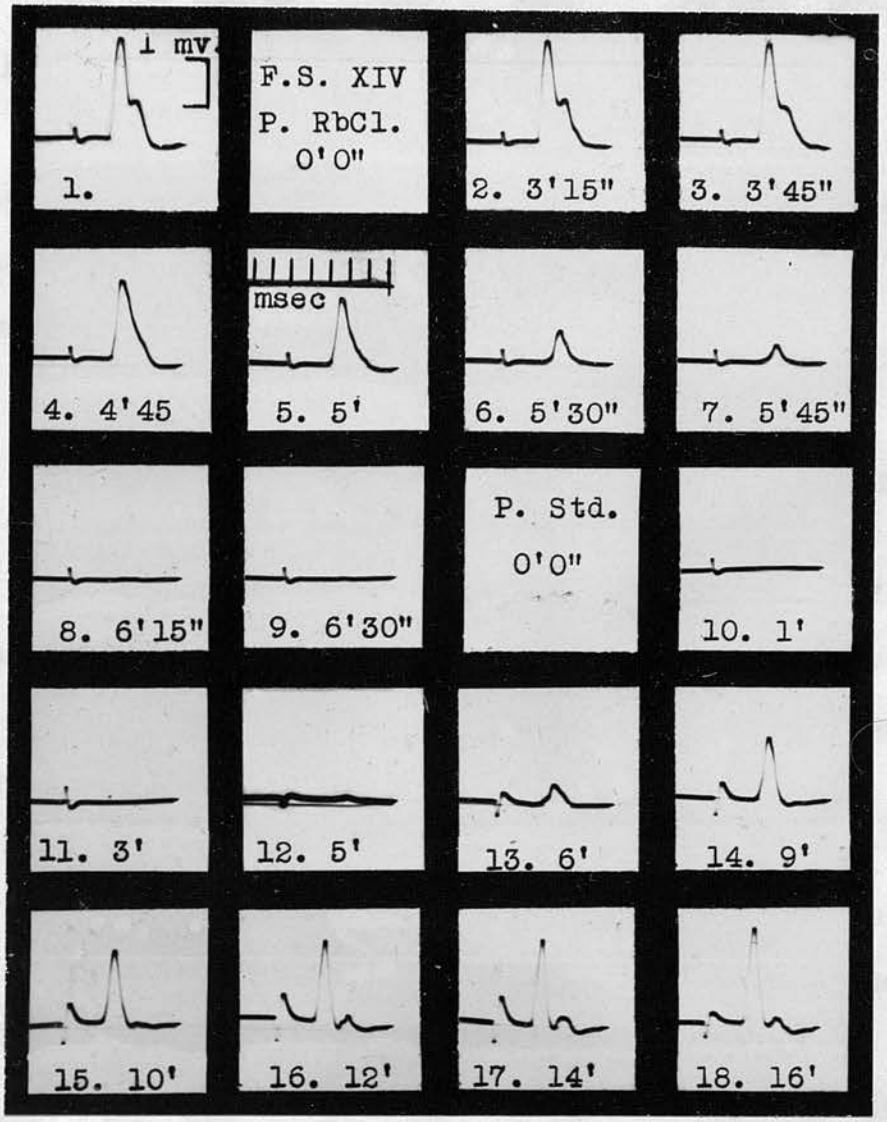

Fig. 7. B. Maximal action potential (with stimulus artefact) of a frog sciatic nerve at various intervals after beginning perfusion with 0.10M RbCl, and then with the standard solution.

No.1 is a normal control, perfused with the standard solution, at the beginning of the experiment.

Read each record from left to right.

b) Perfused nerve (fig.5).

The first sign of a decrease in the action potential appeared within 30 seconds and block was total within 1 - 1½ minutes. Conduction was first seen after some 5 minutes of standard perfusion, and it was normal after some 12 minutes.

ii) 0.10M RbCl.

a) Non-perfused nerve (fig. 6).

The action potential gradually diminished after 5 minutes and conduction ceased after 36 minutes. Recovery began after some 18 minutes and was almost complete after 48 minutes.

b) Perfused nerve (fig.7).

There was no change until after about 4 minutes when conduction began to fail rapidly, so that the block was all but complete in 6 minutes.

The action of both KCl and RbCl always resulted in a rise of the threshold of stimulation.

iii) 0.10M NH_4Cl.

a) Non-perfused nerve.

Block was seen after 50 - 65 minutes.

b) Perfused nerve.

Conduction did not stop until after some 40 minutes.

In neither perfused nor non-perfused nerves was recovery more than very slight and very transient.

iv) Acid Ringer (pH 2.5).

a) Non Perfused (fig.8.)

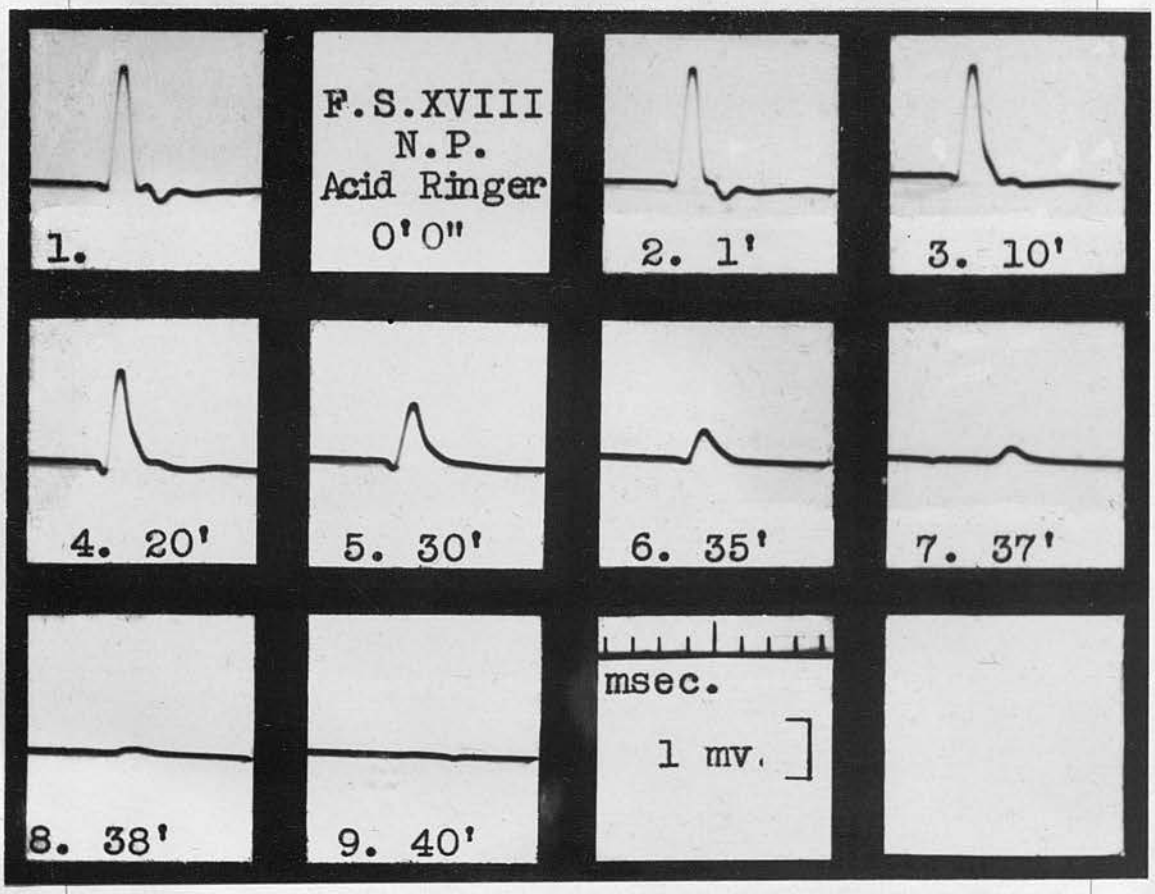

Fig. 8. A. Maximal action potential (with stimulus artefact) of a non-perfused frog sciatic nerve after various intervals in acid Ringer (pH 3.0), and then in the standard solution.

No.1 is a normal control in the standard solution at the beginning of the experiment.

Read each record from left to right.

There was no recovery.

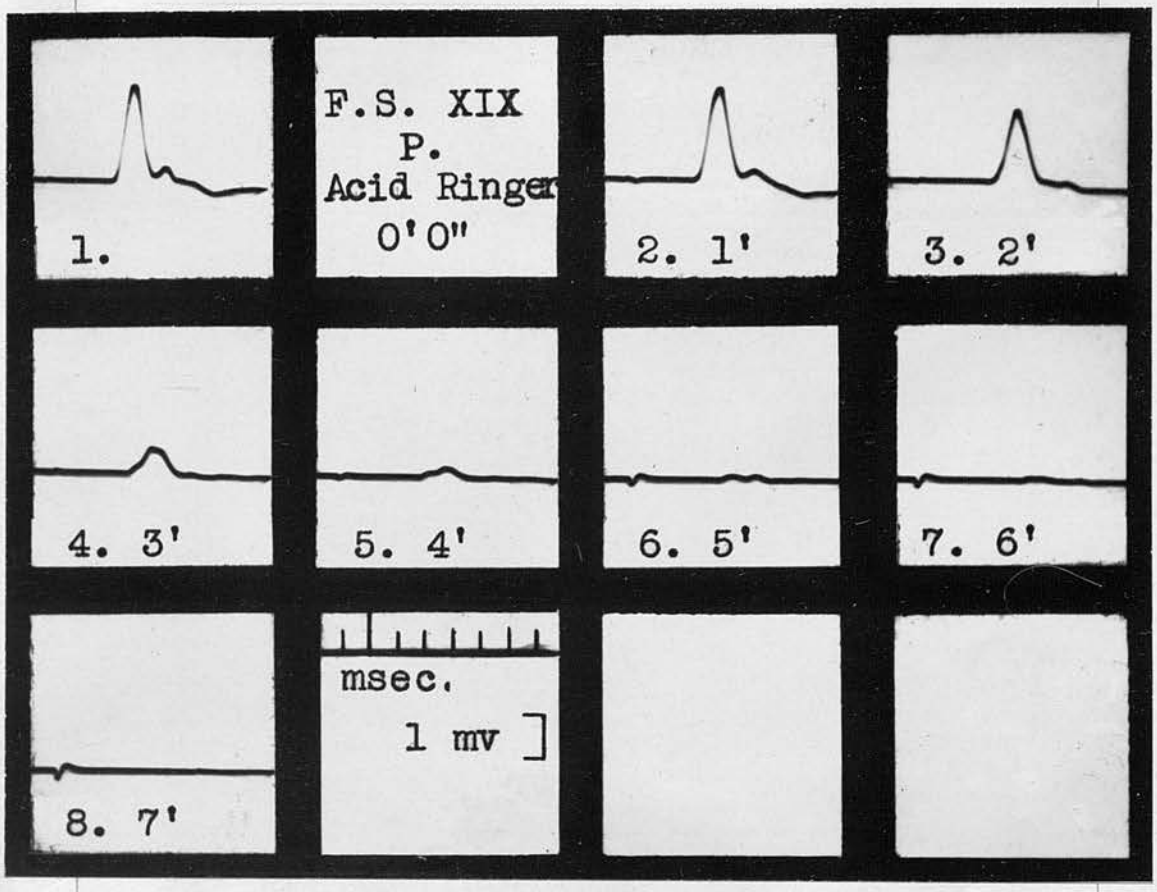

Fig. 9. B. Maximal action potential (with stimulus artefact) of a frog sciatic nerve at various intervals after beginning <u>perfusion</u> with acid Ringer (pH 3.0), and then with the standard solution.

No.1 is a normal control, perfused with the standard solution, at the beginning of the experiment.

Read each record from left to right.

There was no recovery.

The first change was noticeable after 10 minutes and the block was complete at 40 minutes.

b) Perfused nerve (fig.9).

The potential disappeared rapidly so that there was total inexcitability after 6 minutes.

There was no recovery in either case.

v) 0.090M $CaCl_2$.

About 0.011M NaCl was added to prevent interference by Na lack.

a) Non-perfused nerve.

There was a very gradual loss of excitability which became complete after 5 - 6 hours. Recovery in the standard solution was only very slight and transient.

b) Perfused nerve.

The action potential decreased in height after 10 minutes for about 5 minutes, and then remained more or less steady for the next 20 - 30 minutes. Perfusion with the standard solution brought about some recovery, which was not complete, however, after another 30 minutes.

In another experiment the effect of 0.090M CaCl was tried in the absence of Na.

a) Non-perfused nerve.

The gradual change led to complete block after some 7 hours.

b) Perfused nerve.

Conduction ceased after 9 minutes. There was

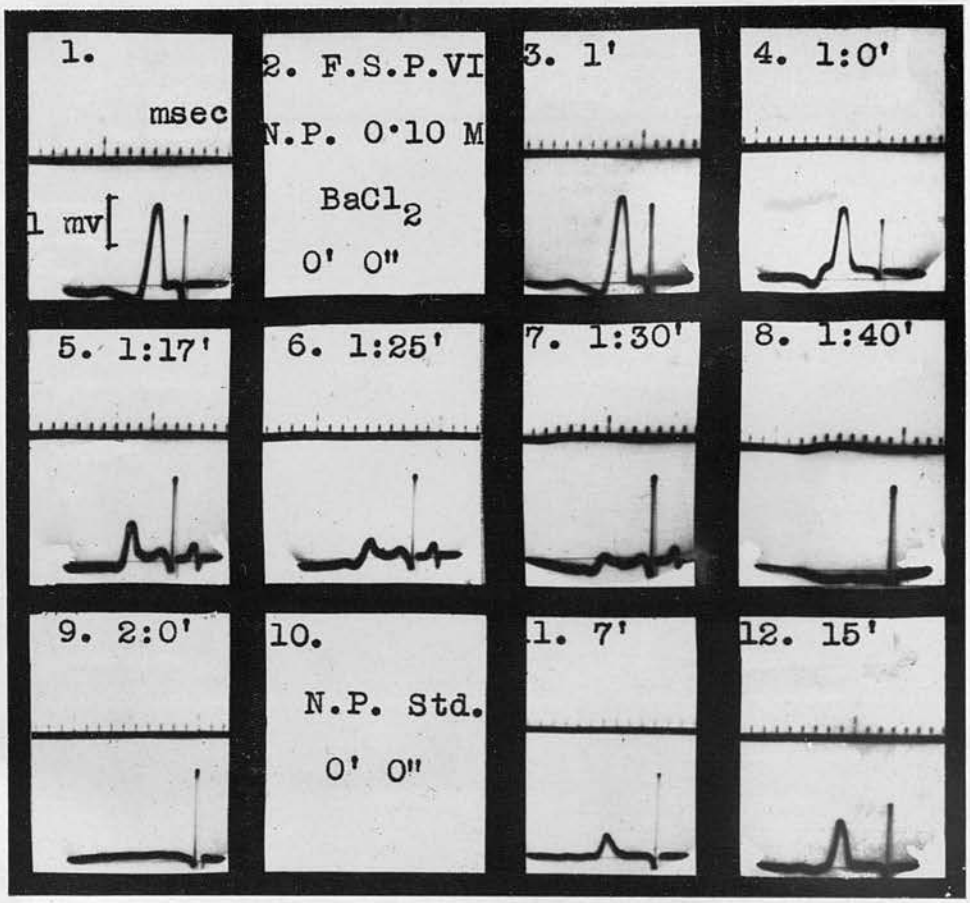

Fig. 10. A. Maximal action potential (with stimulus artefact) of a non-perfused frog sciatic nerve after various intervals in 0.10M $BaCl_2$, and then in the standard solution.

No.1 is a normal control in the standard solution at the beginning of the experiment.

Read each record from right to left.

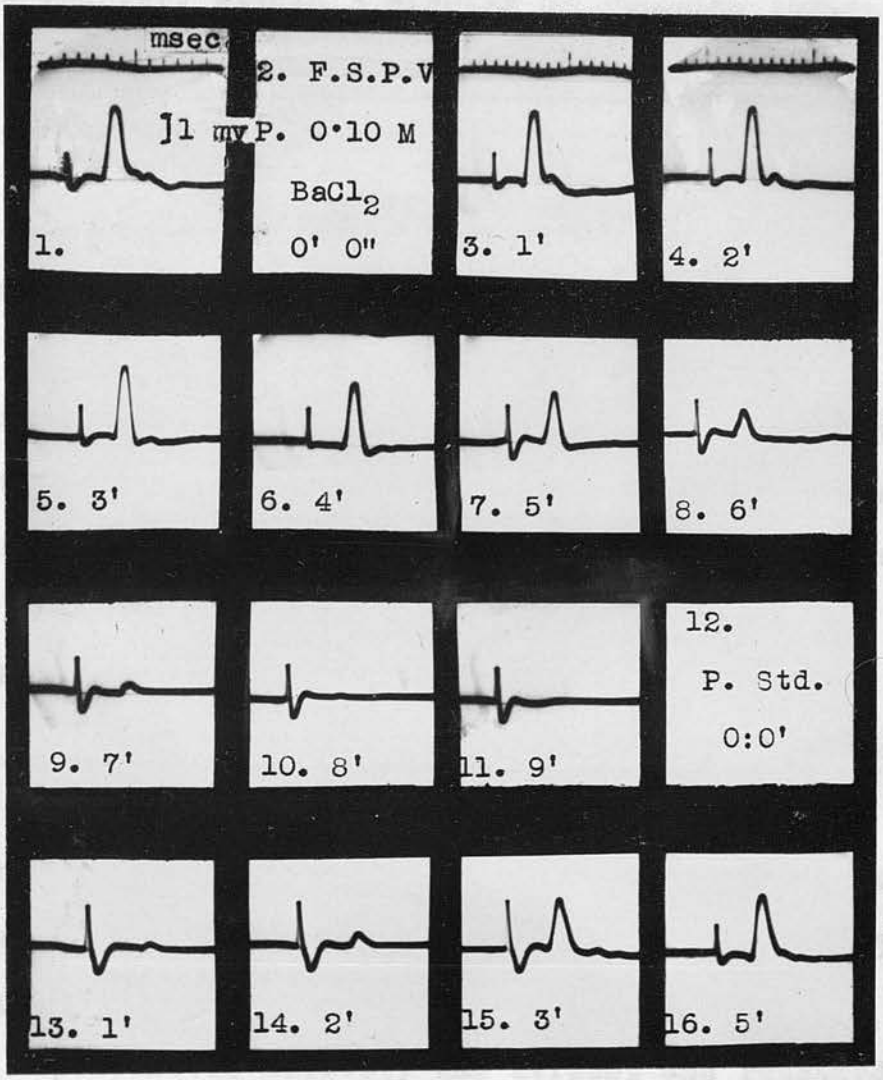

Fig. 11. B. Maximal action potential (with stimulus artefact) of a frog sciatic nerve at various intervals after beginning perfusion with 0.10M $BaCl_2$, and then with the standard solution.

No.1 is a normal control, perfused with the standard solution, at the beginning of the experiment.

Read each record from left to right.

some recovery within 3 minutes of changing the perfusate but there was relatively little change after 6 minutes, and none at all after 20 minutes.

vi) 0.10M $BaCl_2$.

a) Non-perfused nerve (fig.10).

There was definite evidence of increasing inexcitability after 1 hour, and conduction stopped by the end of 2 hours. The first signs of recovery in the standard solution were seen after 6 minutes and further progress was comparatively rapid.

b) Perfused nerve (fig.11).

The action potential diminished rapidly so that the block was complete after 8 minutes. Recovery with the standard perfusion was equally rapid; it began within 1 minute and was nearly complete in 5 minutes.

Spontaneous activity was evident and repeated firing followed single stimuli as a result of the action of Ba.

vii) 0.10M $HgCl_2$.

With powerful corrosive agents like $HgCl_2$ or $CuCl_2$ the ordinary perfusion method cannot be used because the flow rate diminishes extremely quickly, and so the solution never gets as far as the nerve. With a syringe, however a steady flow can be maintained for a few minutes without the rate being increased above usual.

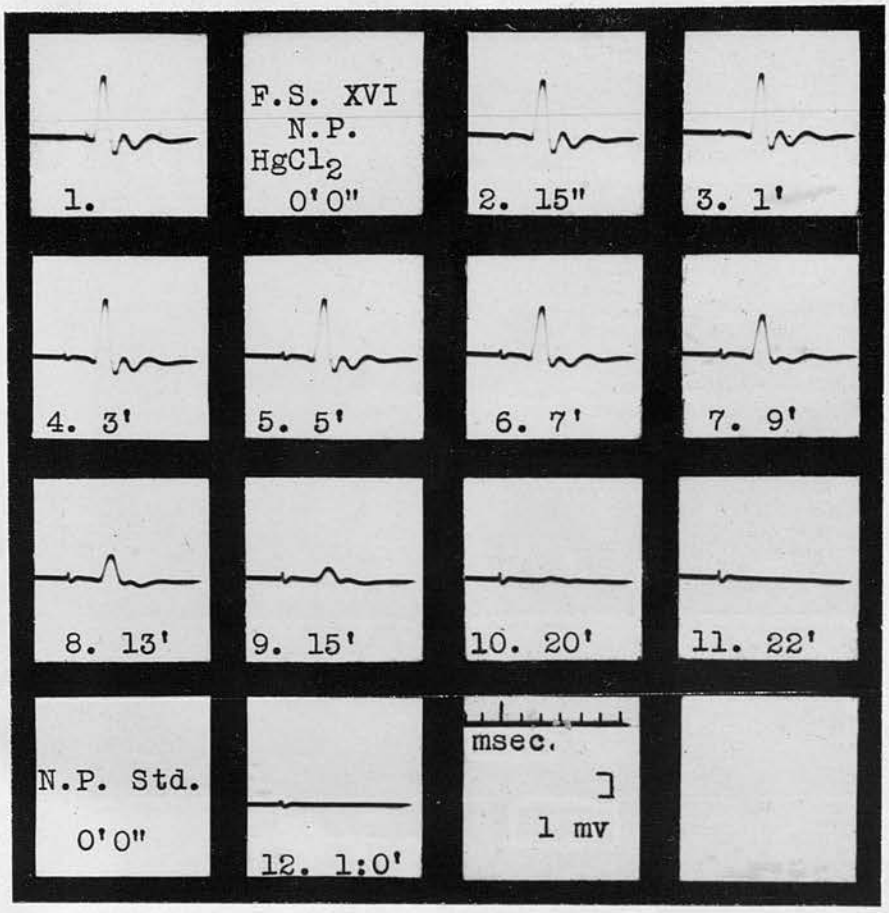

Fig.12. A. Maximal action potential (with stimulus artefact) of a non-perfused frog sciatic nerve after various intervals in 0.10M $HgCl_2$, and then in the standard solution.

No.1 is a normal control in the standard solution at the beginning of the experiment.

Read each record from left to right.

There was no recovery.

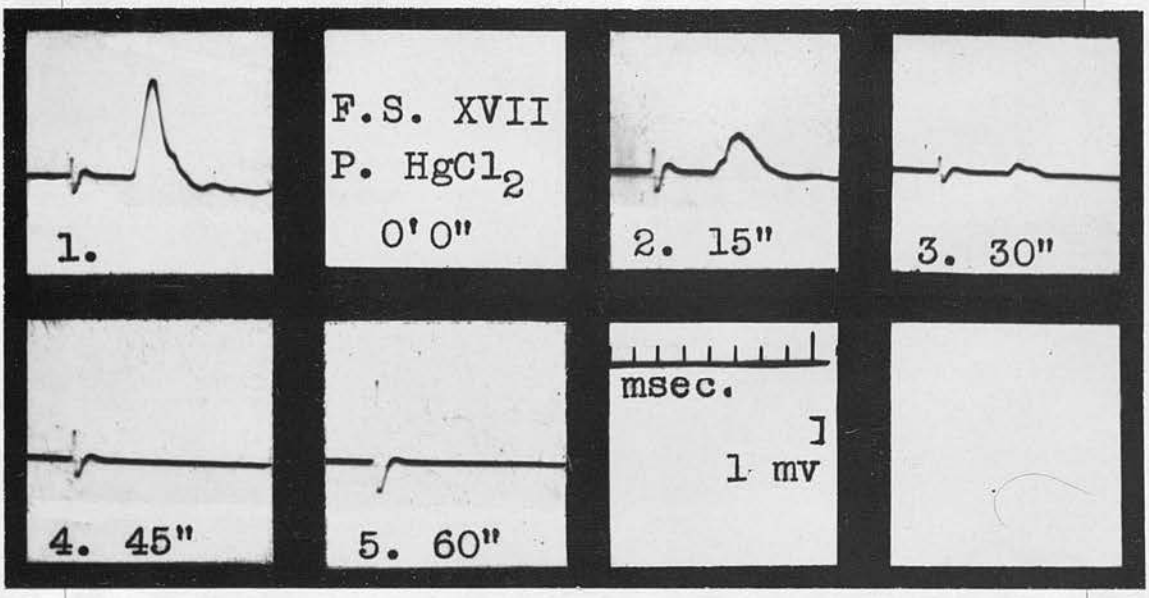

Fig.13. B. Maximal action potential (with stimulus artefact) of a frog sciatic nerve at various intervals after beginning <u>perfusion</u> with 0.10M $HgCl_2$, and then with the standard solution.

No.1 is a normal control, perfused with the standard solution, at the beginning of the experiment.

Read each record from left to right.

There was no recovery.

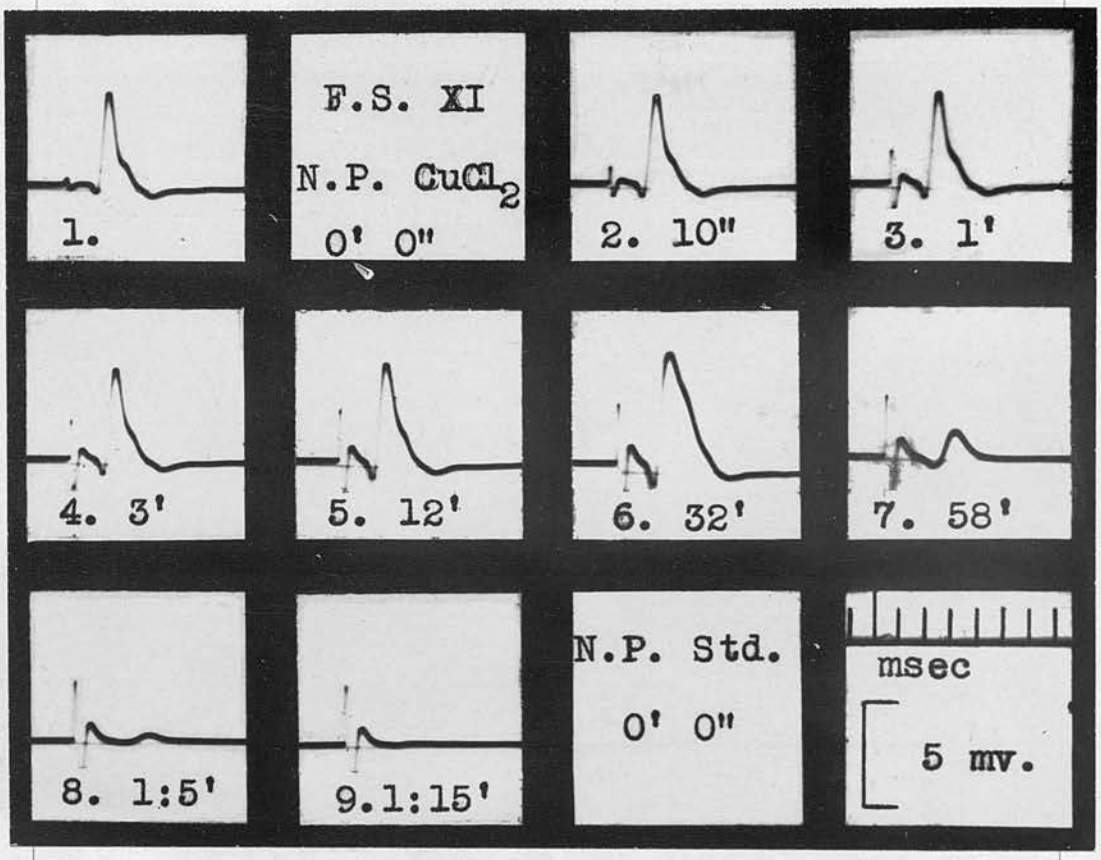

Fig.14. A. Maximal action potential (with stimulus artefact) of a <u>non-perfused</u> frog sciatic nerve after various intervals in 0.10M $CuCl_2$, and then in the standard solution.

No.1 is a normal control in the standard solution at the beginning of the experiment.

Read each record from left to right.

There was no recovery.

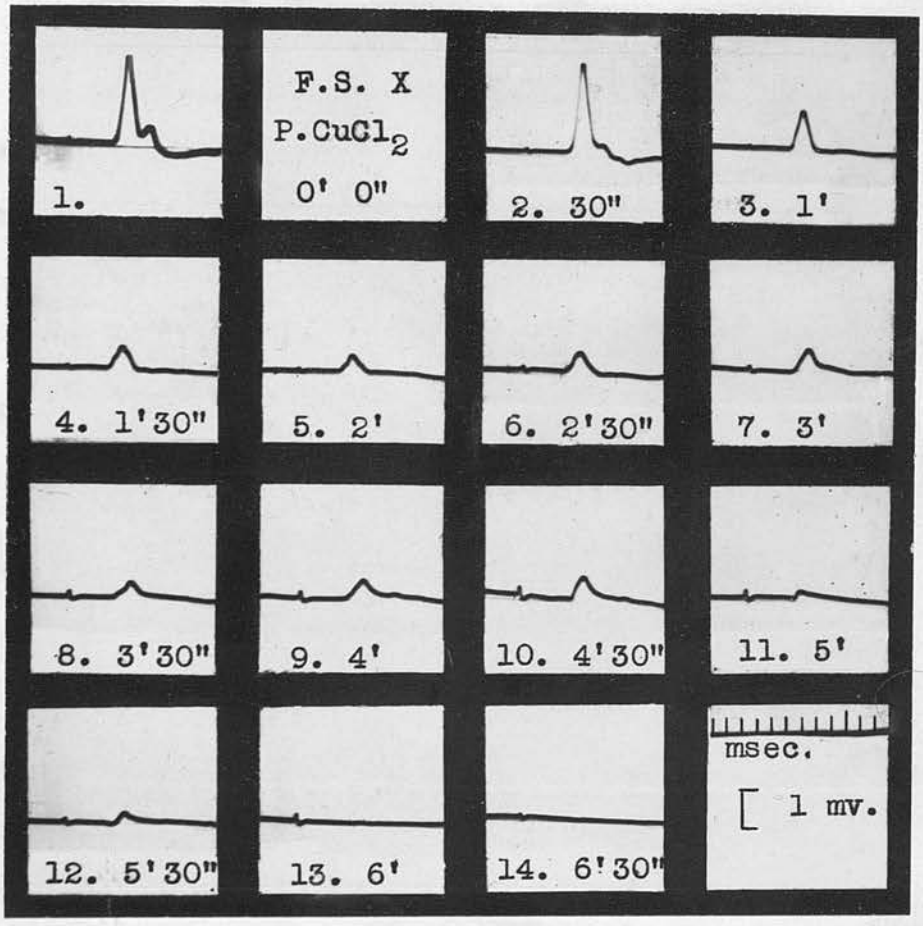

Fig.15. B. Maximal action potential (with stimulus artefact) of a frog sciatic nerve at various intervals after beginning <u>perfusion</u> with 0.10M $CuCl_2$, and then with the standard solution.

No.1 is a normal control, perfused with the standard solution, at the beginning of the experiment.

Read each record from left to right.

There was no recovery.

a) Non-perfused nerve (fig.12).

Conduction was not affected for the first 5 minutes and it did not cease altogether until 22 minutes had passed.

b) Perfused nerve (fig.13).

The effect was practically immediate, and it was complete within 45 seconds.

No recovery was seen in either a) or b)

viii) 0.10M $CuCl_2$.

a) Non-perfused nerve (fig.14).

Little change was seen for the first 15 - 20 minutes; there was then a rather striking increase in the size of the potential, followed by a comparatively rapid decline leading to full inexcitability after about 1¼ hours.

b) Perfused nerve (fig.15).

After 30 seconds, the potential diminished very quickly but it then fluctuated more or less evenly up and down over the next 3 minutes; at about 5 minutes it decreased again, still fluctuating, and disappeared at about 6 minutes.

There was no recovery in either a) or b).

3. <u>The effect of lipoid soluble substances.</u>

i) 10% (v/v) acetone in standard solution.

a) Non-perfused nerve (fig.16).

The action potential had obviously decreased after 1 minute, and it disappeared after 2 minutes.

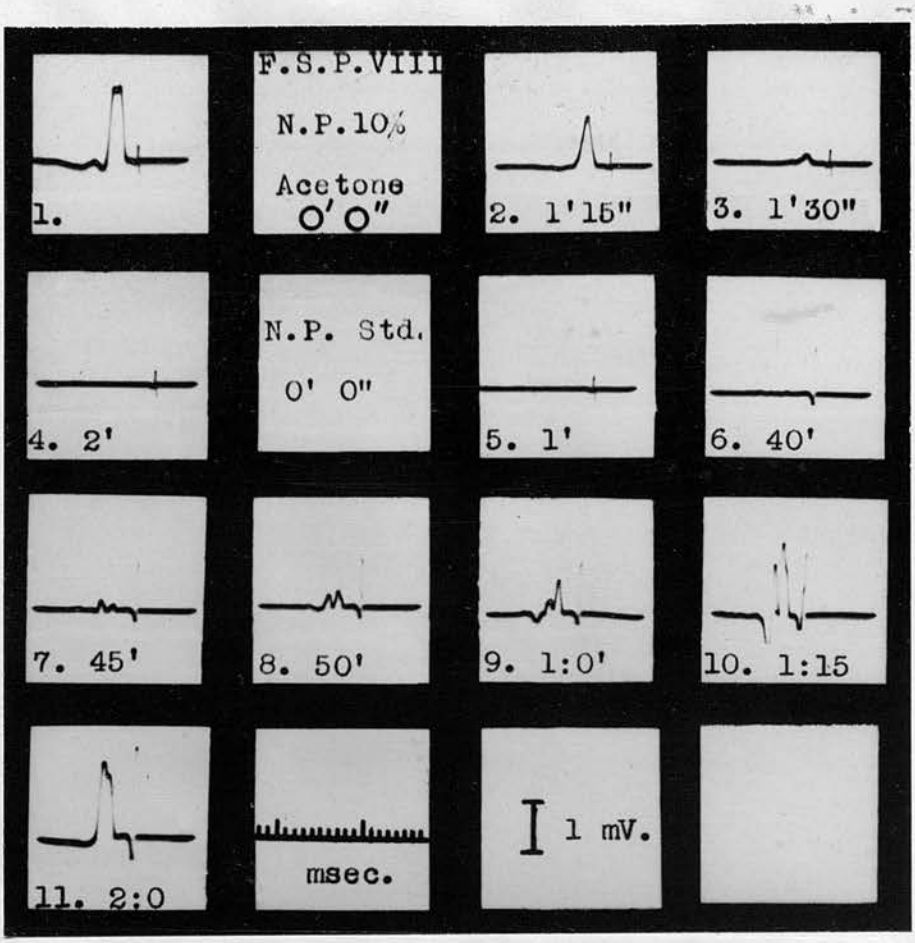

Fig.16. A. Maximal action potential (with stimulus artefact) of a non-perfused frog sciatic nerve after various intervals in 10% (v/v) acetone, and then in the standard solution.

No.1 is a normal control in the standard solution at the beginning of the experiment.

Read each record from right to left.

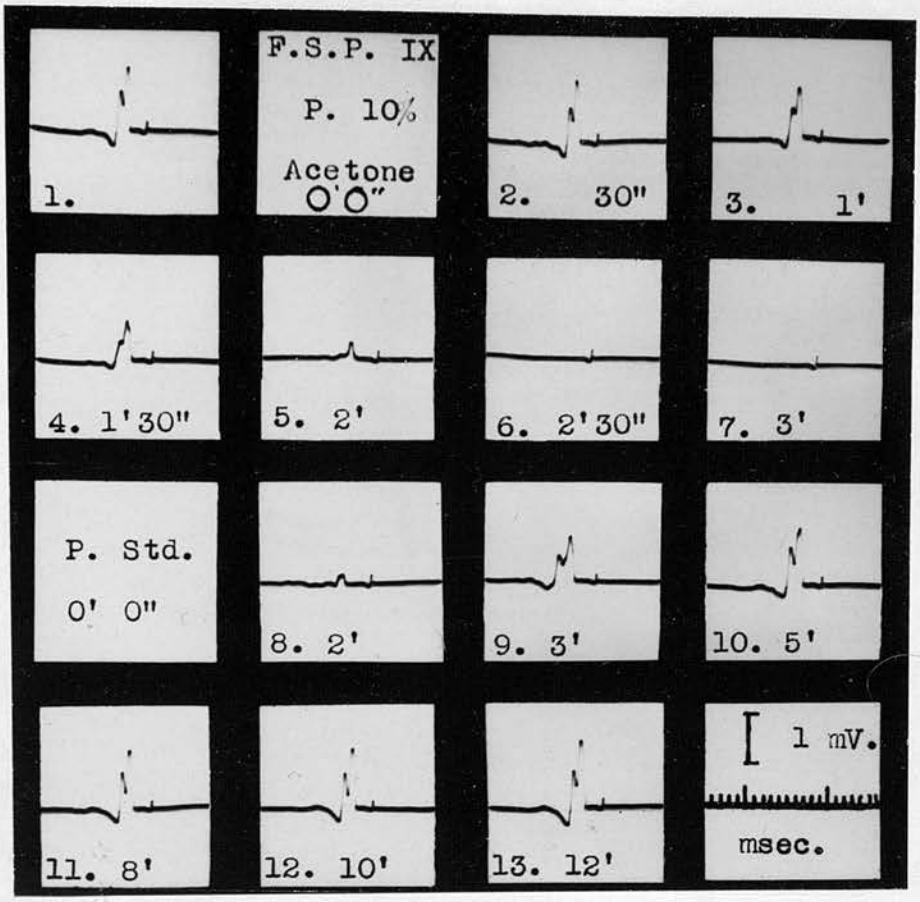

Fig.17. B. Maximal action potential (with stimulus artefact) of a frog sciatic nerve at various intervals after beginning perfusion with 10% (v/v) acetone, and then with the standard solution.

No.1 is a normal control, perfused with the standard solution, at the beginning of the experiment.

Read each record from right to left.

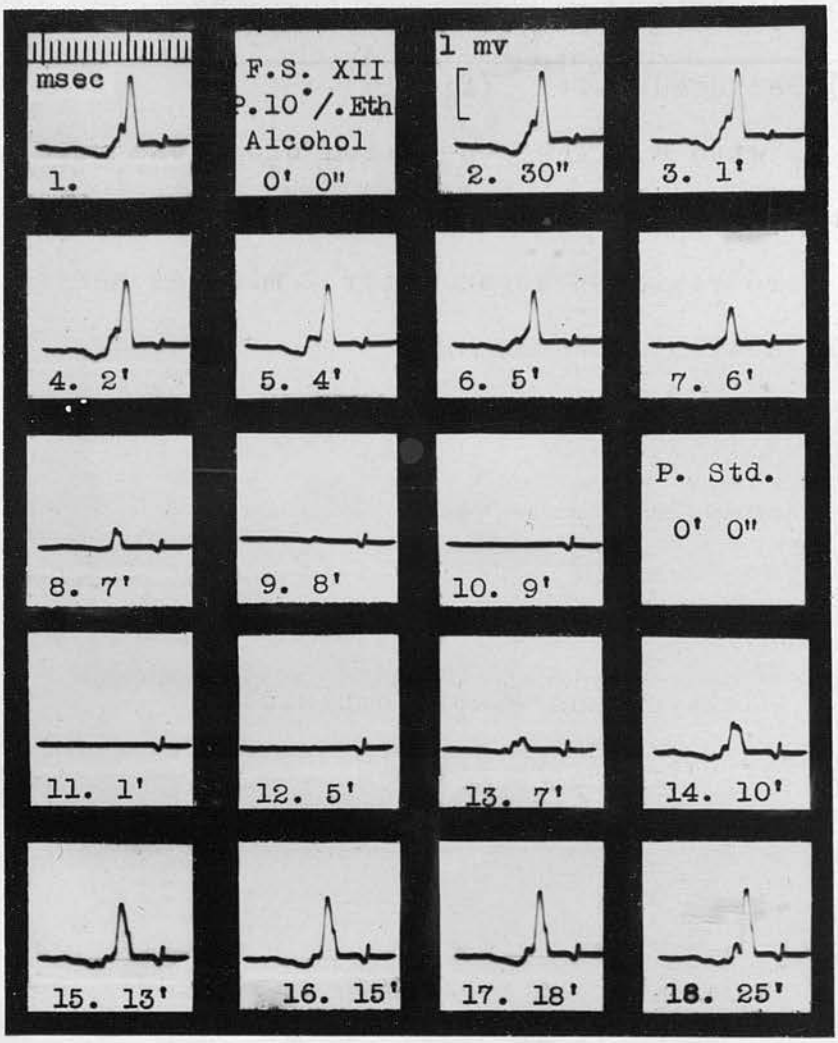

Fig. 18. B. Maximal action potential (with stimulus artefact) of a frog sciatic nerve at various intervals after beginning perfusion with 10% (v/v) ethyl alcohol, and then with the standard solution.

No.1 is a normal control, perfused with the standard solution, at the beginning of the experiment.

Read each record from right to left.

Recovery only began after 40 minutes but was nearly full after 2 hours.

b) Perfused nerve (fig.17).

As with a), the conduction block was absolute in about 2 minutes. Recovery, however, was very much more rapid; it began after 2 minutes and was complete within 12 minutes.

ii) 10% (v/v) ethyl alcohol.

a) Non-perfused nerve.

Inexcitability was rapid in onset; it was complete in about 6 - 7 minutes. Recovery was greatly delayed and was incomplete.

b) Perfused nerve (fig.18).

The block was not complete for some 8 - 9 minutes, but recovery was nearly full in 25 minutes.

50% ethyl alcohol was also tried in one experiment; the control nerve maintained conduction for about 2 - 3 minutes, but the injected nerve stopped conducting after 1 minute.

iii) 0.015M cocaine HCl.

a) Non-perfused nerve.

The blocking times varied between 3 - 11 minutes and recovery was slow and only partial.

b) Perfused nerve.

The blocking times here tended to be higher than in a); the range covered was between 12 - 25 minutes. Recovery was only imperfect.

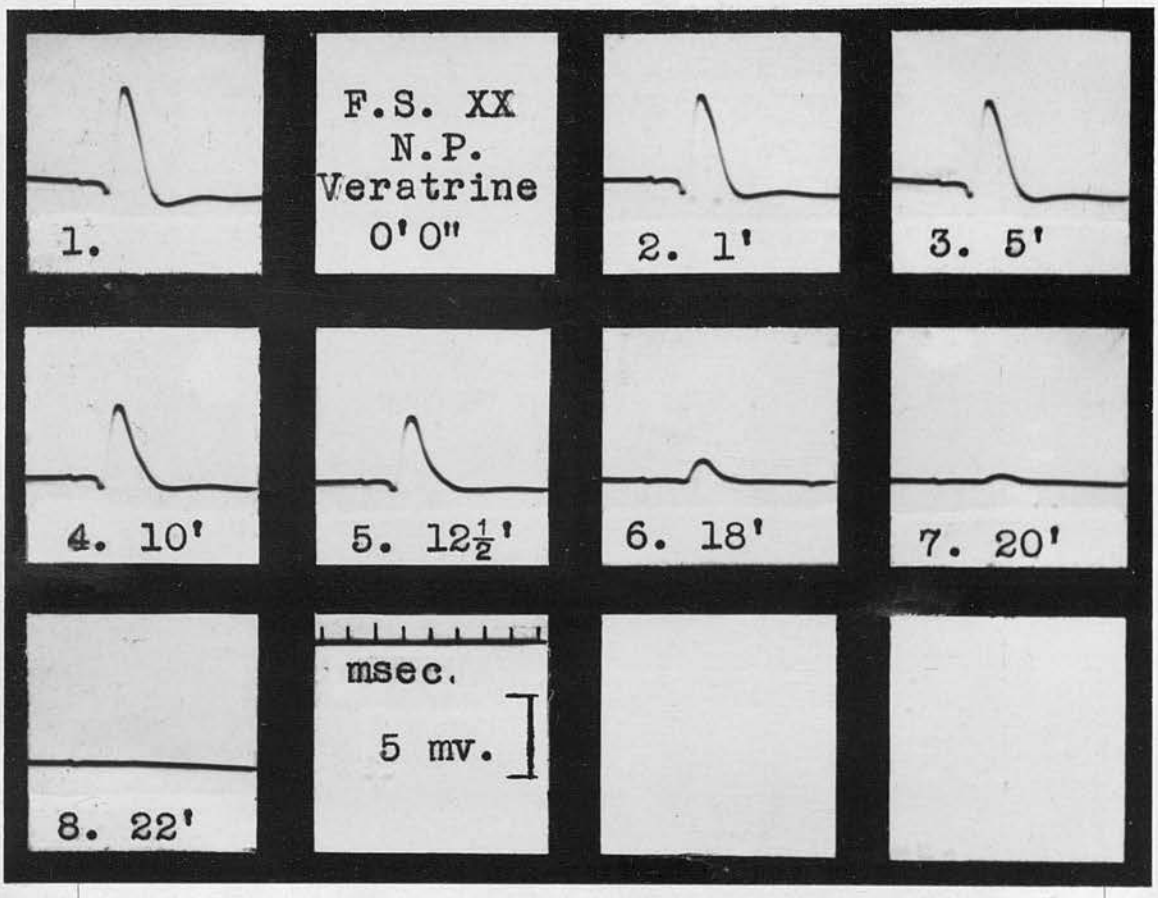

Fig.19. A. Maximal action potential (with stimulus artefact) of a non-perfused frog sciatic nerve after various intervals in 1/5000 Veratrine HCl, and then in the standard solution.

No.1 is a normal control in the standard solution at the beginning of the experiment.

Read each record from left to right.

There was no recovery.

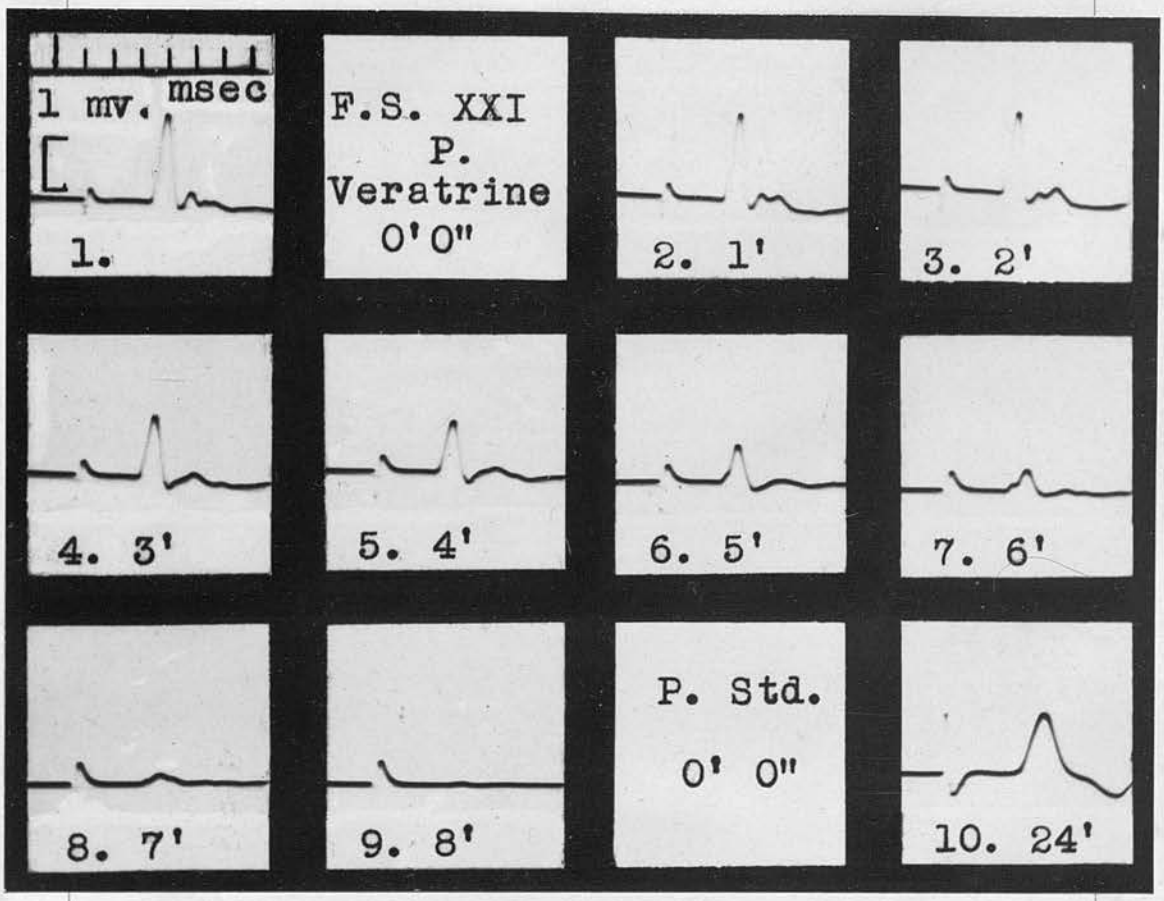

Fig.20. B. Maximal action potential (with stimulus artefact) of a frog sciatic nerve at various intervals after beginning perfusion with 1/5000 Veratrine HCl, and then with the standard solution.

No.1 is a normal control, perfused with the standard solution, at the beginning of the experiment.

Read each record from left to right.

It should be pointed out that the frogs employed were mostly in a rather poor condition; the blocking times of symmetrical non-perfused nerves differed by as much as 100%. In a carefully controlled experiment, with a vigorous, apparently well-fed frog, the non-perfused and perfused blocking times were 11 and 12 minutes respectively.

iv) 1/5000 veratrine HCl.

a) Non-perfused nerve (fig.19).

The action of the alkaloid was quite rapid: conduction ceased after 22 minutes. There was no recovery.

b) Perfused nerve (fig.20).

The action potential diminished quickly and disappeared after about 8 minutes. There was only partial recovery in the standard solution.

Desheathed nerves.

A number of frog sciatic nerves were desheathed in the course of some investigations into the effect of various degrees of Na deficiency upon the conduction velocity and the refractory period. The rate at which inexcitability occurred in a Na free medium was observed in some cases, and these are presented below since they are relevant to the subject of this thesis.

Na substitute	Blocking time	Initial recovery time
1. Sucrose	25 min	20 sec
2. Choline chloride	9 min	Not recorded
3. Choline chloride	5 min	40 sec
4. Choline chloride	3 min	40 sec
5. Sucrose	2½ min	20 sec
6. Choline chloride	45 sec	30 sec
7. Choline chloride	15 sec	15 sec

Nerves 6 and 7 were phalangeal preparations which had been desheathed down to the level of the ankle, and in which only two units were functioning.

Nerve 1. was one of the first preparations, in which desheathing was probably incomplete.

The recovery times only give a rough indication of the rate of recovery, as in previous experiments.

Intraneural injections.

The purpose of these injections was to study the longitudinal movement of fluids under pressure within a nerve and also the possibility of trans-perineural drainage.

When 1/1000 methylene blue (in Ringer) was injected into the sciatic nerve simply with a syringe, the dye (0.2 cc.) passed up about 1.2 cm relatively easily within 2 - 3 minutes; but progress along the cords of the lumbosacral plexus seemed much slower. There was no spread outward to the surface.

The injection was repeated under controlled pressure in six nerves with the following results:

i) Longitudinal flow was extremely slow at all pressures up to about 200 to 300 mm. Hg; for instance at 10 - 20 mm. Hg. the dye penetrated no more than 1 mm. after 5 minutes; even at 100 mm.Hg. the penetration was only some 4 - 5 mm. At 200 mm Hg. about 0.5 cm. was the distance passed in 5 minutes and even after 40 minutes it was only 1.2 cm. A pressure of 300 mm.Hg. filled 1.5 cm. in about 10 minutes. This meant that the sciatic nerve was stained with the dye up to its origin in the lumbosacral plexus, the cords of which were stained for a few millimetres, but further progress was very slow. Even after 60 to 90 minutes at a high pressure (200 - 300 mm. hg.), the dye did not reach a point less than 1 cm. from the spinal column, but

branches of the sciatic nerve, such as the nerve to the hamstrings, were stained by methylene blue which travelled in a peripheral direction for about 1 cm.

ii) The outward escape of methylene blue was tested by applying fine moist strips of filter paper to the surface of the nerve at intervals and comparing them with control strips. When escape did occur, external staining was quickly abundant; it was found, however, that the pressure necessary to produce rapid exudation was never less than 200 - 300 mm. hg. At 100 mm Hg. there is distinct swelling of the nerve, but even after 20 minutes, no dye was detected at the surface.

The results obtained with centrifugal intra-neural injections were comparable as far as the longitudinal movement is concerned, but in one case, some methylene blue was detected at the surface of the nerve after 10 minutes of injection at only 20 mm. Hg. It is not clear whether this was caused by injury to the perineurium, or whether it is a genuine example of relatively high permeability. This isolated observation suggests the former rather than the latter.

Histological sections of the injected nerves showed that the methylene blue is not distributed evenly. At the site of injection, and for a few millimetres further up, the dye is found throughout

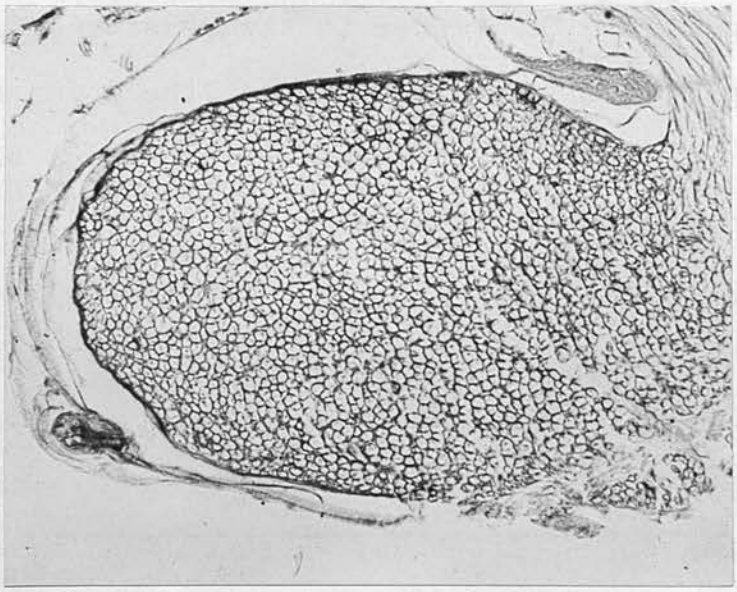

Fig.21. Transverse section of a frog sciatic nerve, about 1 cm. from the site of an intraneural injection of 1/1000 methylene blue. The dye can be seen in, and immediately under, the perineurium x 140.

the bundle between the axons. As the distance increases, however, the dye is only to be seen at the periphery, immediately under or within the perineurium (fig.21). The latter is always stained rather strikingly by the methylene blue; the point of furthest penetration is shown by very faint blue colouring of the perineurium.

It will have been noticed that injection at a high pressure for over one hour failed to stain the proximal part of the lumbosacral plexus, so that no light was thrown on the problem of a neuro-subarachnoid pathway. A different approach was therefore tried: a canula was introduced into the spinal canal in the upper sacral region. When methylene blue was injected by means of a syringe, there was obvious staining of the lumbosacral plexus, but an injection at 15 mm. Hg. for 35 minutes produced only faint colouring. The interpretation of this must be left open because some dye had been widely disseminated in the vascular system (probably via arachnoid granulations cf. Leonard Hill quoted by Weed, 1914) and microscopic confirmation was not obtained owing to a technical mishap.

Blood nerve barrier.

All the tests of the hypothetical nerve barrier were based upon the dye technique which has been used so often in investigations of the blood brain barrier. No quantitative results were obtained in

these experiments since the method allows only a relatively rough and ready estimation of the distribution of dyes in tissues.

The frogs were perfused with :

0.1% aniline blue in Ringer.

0.1% and 1% acid fuchsin in Ringer.

1% iron ammonium citrate and K ferricyanide.

There was always general but uneven staining of the tissues. The abdominal viscera were usually deeply coloured, but the muscles of the limbs were always rather lightly stained. The testes were conspicuously unstained, and so were the brain and the spinal cord. The peripheral nerves usually stood out against the darker background of the surrounding tissues although they were apparently stained to some extent. After perfusion with the prussian blue reagents for 30 minutes, at 25 - 30mm. Hg. sections of the sciatic nerves were compared with sections of one of the thigh muscles and of a piece of gut. The blue stain was present in most parts of the nerve around the nerve fibres, and apparently in a concentration not very much less than in the muscle; however, the central nervous tissues were also stained blue so that the only conclusion that may be reached is that there was relatively little differentiation between various tissues in their permeability to the prussian blue reagent under those conditions.

In ~~an~~ one experiment, 1.5 cc. of 0.25% aniline blue were injected subcutaneously and the animal left overnight. As the final concentration of the dye was relatively low, the results were even more inconclusive.

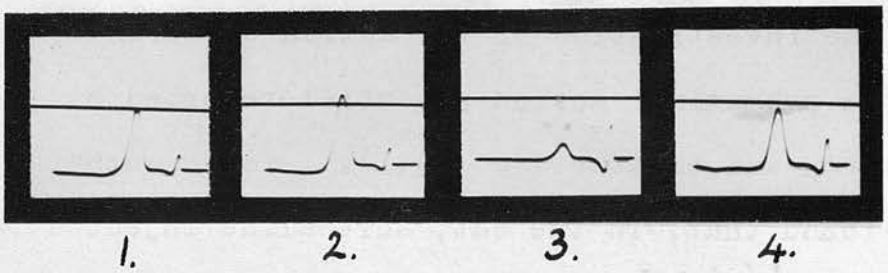

Fig.22. Actions of certain drugs upon submaximal action potential of a perfused frog sciatic nerve. Read each record from right to left. The upper trace is fixed at the height of the control potential (1.) which was 75% maximal.

2. 2 Minutes after injecting 0.1 mg. adrenaline into the perfusate.

3. 1 minute after injecting 3 cc. of a solution obtained by shaking 3 minims (approx.0.18 cc.) amyl nitrite in 50 cc. Ringer.

4. 1 minute after injecting 1/3 unit of a posterior pituitary extract.

The very large doses injected were exceptional; they were necessary as the nerve had become very unresponsive as a result of previous injections.

The action of adrenaline on the submaximal action potential of the perfused frog nerve.

The perfused nerve preparation seemed suitable for the investigation of the action of adrenaline on the submaximal action potential reported by Bülbring and Whitteridge (1941). These authors had found that, in the cat, adrenaline injected intra-arterially has the effect of increasing the size of the submaximal action potential recorded from a branch of the sciatic nerve, and they suggested that adrenaline lowers the threshold of excitation of the nerve fibres.

These experiments were repeated with perfused frog nerves. Adrenaline was injected into the rubber tubing close to the canula. Stimulation was submaximal and a high resistance (100,000 ohms) was placed in series with the nerve to maintain the total current passing through the nerve approximately constant (as did Bülbring and Whitteridge). The nerve was crushed between the recording electrodes as usual.

In preliminary experiments it was found that the effect described above could be reproduced. However, it was also found that the potentiating action of adrenaline was shared by extracts of posterior pituitary, and that it was reversed by vasodilator drugs such as Na nitrite and amyl nitrite (fig.22).

It was then thought likely that these phenomena

Fig.24. Transverse section of the pelvic portion of a frog sciatic nerve perfused with prussian blue in gelatin and fixed in absolute alcohol. x 95.

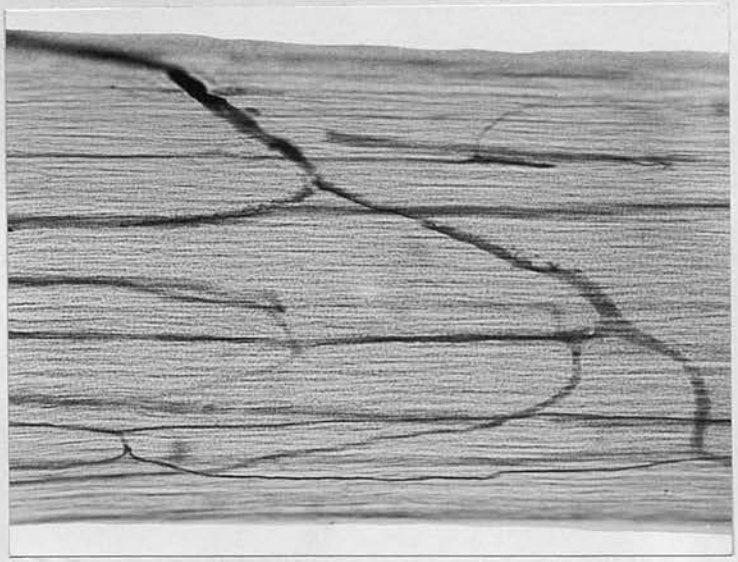

Fig.25. Low power view of the mid-thigh portion of a frog sciatic nerve perfused with prussian blue in gelatin, fixed in absolute alcohol and mounted whole in damar after clearing in xylol. Only those vessels are seen which are more or less in focus. x 100.

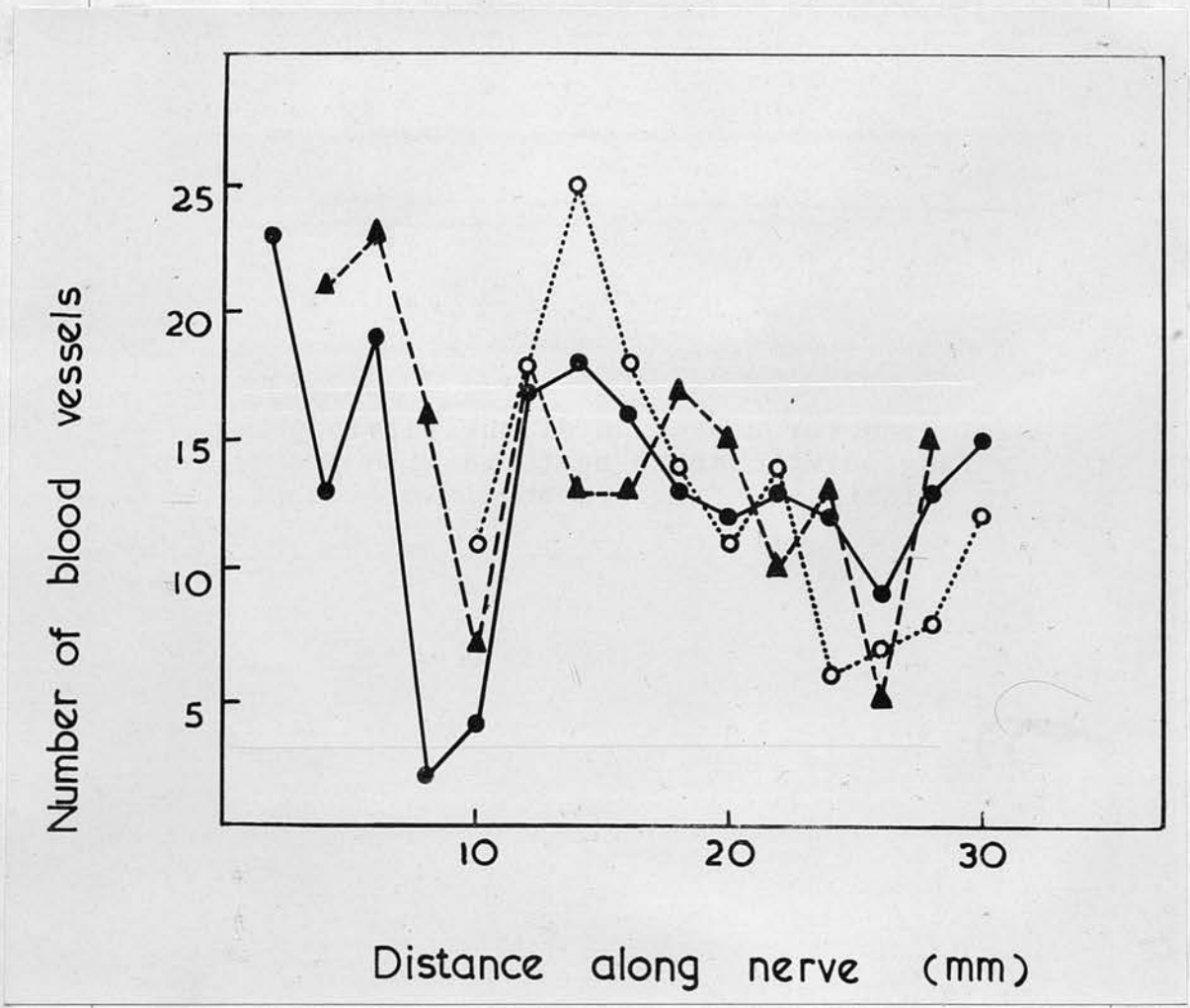

Fig.26. Total counts of vessels in 3 perfused frog sciatic nerves at intervals of 2 mm. The point of reference for comparison of the 3 curves is the terminal sciatic bifurcation placed arbitrarily between 28 and 30 mm.

were caused by changes in the extracellular fluid content of the nerve, which should produce corresponding variations in the penetrating fraction of the constant stimulating current. This hypothesis was tested by measuring the total impedance between the stimulating electrodes in a Wheatstone bridge, at intervals after injecting adrenaline.

The size of the submaximal potential varied spontaneously, but not without corresponding fluctuations in the interelectrode impedance. The effect of 5μg and 1μg. of adrenaline on the action potential and on the interelectrode impedance is shown in graph form in fig. 23. There is not exact correspondence between the two curves, but this cannot be expected as measurements of the impedance could not be made instantaneously, and so there was often some unavoidable lag between the two readings. However, the general agreement in the shapes of the two curves seems satisfactory. It is rather interesting that there was a pronounced rebound effect after the first dose of adrenaline, which was only just neutralised by the second, smaller dose.

One cannot avoid the conclusion that, in the perfused frog nerve at any rate, the apparent potentiating action of adrenaline is the result of changes in the total fluid content of the nerve, and not a real increase in excitability.

Counts of blood vessels in frog sciatic nerves.

Counts were made of vessels with the low power of a microscope in nerves perfused with prussian blue in gelatin or Indian ink. The portion of nerve studied extended for about 6 cm. from the pelvis to the ankle and included cords of the lumbosacral plexus, the sciatic nerve and the peroneal nerve.

Two groups of studies were made:

1) Vessels were counted in histological sections (fig.24). The dimensions of the nerve were also recorded so that an estimate could be made of the relative concentration of the vessels.

The distribution of vessels in 340 counts in six nerves was by no means even. Values ranged from 0 to 150/mm^2. The higher figures (100 or over) were associated particularly with a few fine branches (diameter = 0.16 mm. or less). The very low figures (under 10) were seen, of course, in nerves in which the perfusion had failed conspicuously, but even in the best specimens, there were usually some very low counts, (about 10% of the total). It has not been possible to determine exactly to which extent these are the result of poor filling; however, there is reason to believe that the apparently avascular regions are not distributed at random along the nerve but are to be found in corresponding regions of different nerves. This is shown more clearly by the counts made in whole nerves described later under (2).

The mean concentration of vessels in the nerves examined histologically was 45/mm^2. A better indication of the vascularity of any one nerve is given by figures for some typical cases:

	Mean	Standard deviation
(number of vessels/mm^2)	62	28
	53	20
	49	15
	35	25
	31	21.7

The difficulty of obtaining a full vascular injection in the frog muscle was stressed by Krogh (1919 b). In muscle, it is relatively easy to allow for deficient filling by selecting suitable sites for the counts; nevertheless, Krogh found that the concentration of vessels varied between 300 and 500/mm^2. The association of low values of the mean with high values of the standard deviation is undoubtedly due to the presence of a higher number of apparently avascular patches. If allowance is made for values less than 10/mm^2. then the overall mean is about 51 vessels/mm^2.

The vessels are usually distributed regularly within any one cross-section of the nerves, as can be seen in fig.24. Small nerves, with a diameter less than 0.2 mm. or so, tend to have a relatively large central vessel which may or may not be accompanied by one or two finer branches. There is no obvious change in the relative concentration along the

nerve as a function of the diameter until the finer branches are reached, as already mentioned.

The exact measurement of the dimensions of the vessels in this type of injection experiment is rather difficult, as Krogh (1919 b) pointed out, but approximate figures were obtained by measuring the diameter of a little over 200 vessels in two nerves perfused with prussian blue and gelatin. The average diameter was 9.5 μ , and the range covered was 3.5 - 28 μ . Of the total, only 10 diameters exceeded 14 μ and of these only 4 were greater than 18 μ . The average diameter of the frog muscle capillaries observed by Krogh, (1919 c) was apparently only about 5 - 7 μ , varying with the condition of the muscle (i.e. whether at rest or stimulated). However, Zweifach's (1937) measurements of frog capillaries gave substantially greater diameters of the order of 9 - 12 μ , It may therefore be assumed that the great majority of the vessels counted were in fact capillaries; whether the larger vessels (20 μ or over) are functionally capillaries, or arterioles and venules, is rather difficult to decide.

2). The second type of observation was made upon nerves which, after perfusion with prussian blue in gelatin or Indian ink, were dehydrated, cleared, and then mounted whole on slides. These preparations have the advantage of presenting the vessels as a

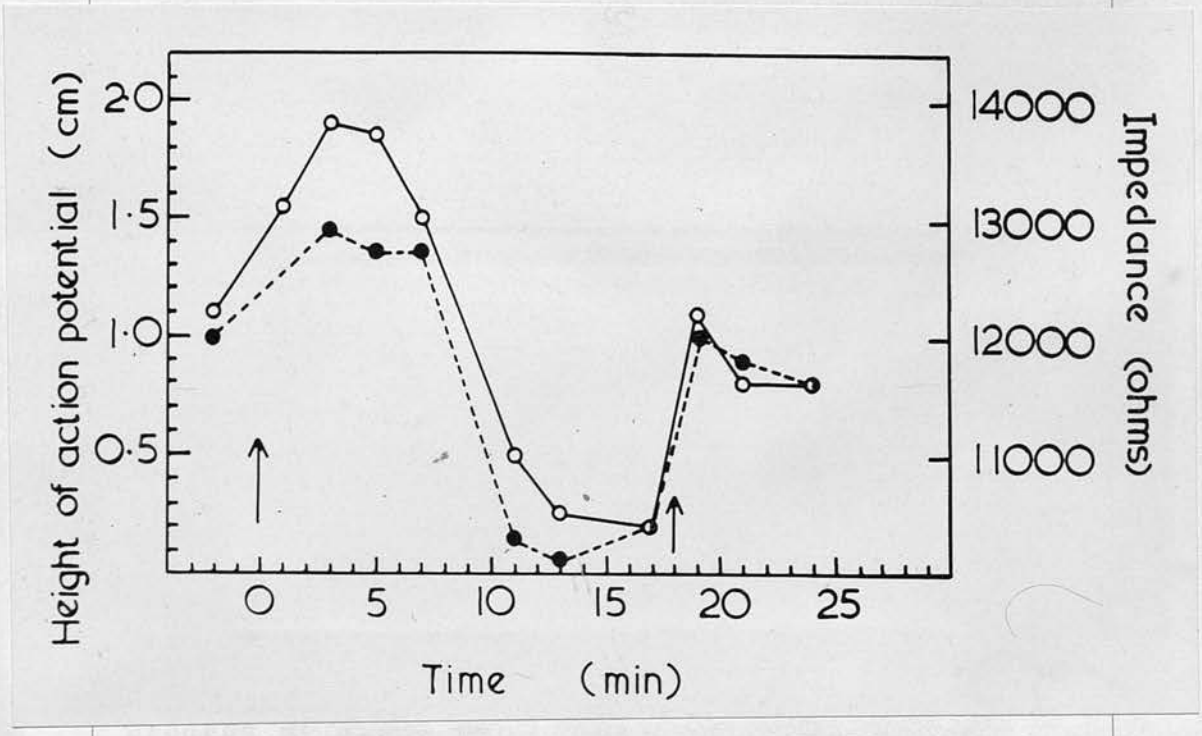

Fig.23. Relationship between height of submaximal action potential ( ) and nerve impedance between stimulating electrodes ( ). At the first arrow, 5 adrenaline were injected, and 1 at the second. Height of maximal potential was 2.05 cm.

continuous system which can be followed in all its ramifications both longitudinal and transversal. Fig. 25 illustrates clearly the very characteristic loops formed by an arteriole, which has penetrated into the nerve, as it branches rapidly in a series of parallel capillaries that run longitudinally along the fibres. They anastomose with other similar capillaries, and also send transversely some intercommunicating channels.

This method gives a faithful account of the vascular filling in the nerve but it does not allow one to make exact measurements of the nerve itself which is deformed to an unknown degree by the process of mounting. However, a comparison was made of nerves examined by the two methods at corresponding points: the total counts of vessels were found to be essentially similar.

Some 180 counts of vessels were made at regular 1 or 2 mm. intervals along whole nerves; from these it became obvious that highly vascular portions alternated with others which were more or less avascular. At the site of an important bifurcation, there was always a relatively high number of vessels, while the immediately preceding stretch of nerve was noticeably deficient in this respect. This could often be seen even with the naked eye, as alternating lighter and darker patches. The upper half of the specimens (i.e. the sciatic nerve and its roots) showed this much more clearly

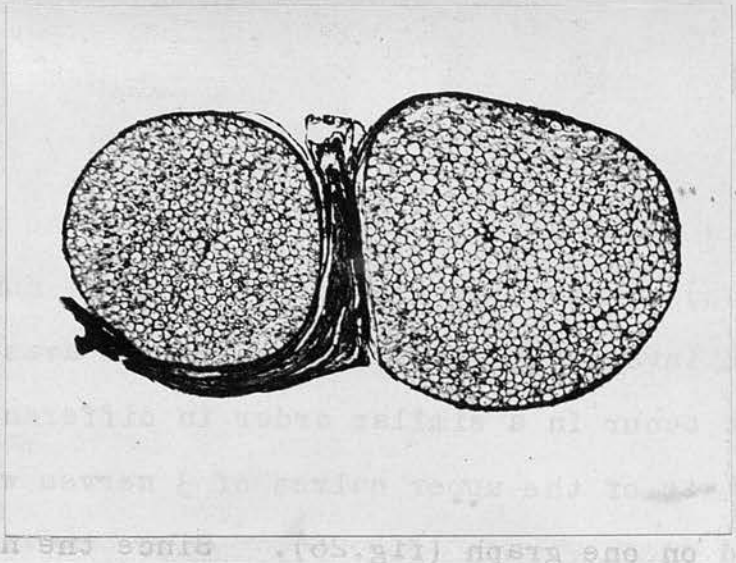

Fig.27. Transverse section of a frog sciatic nerve at its terminal bifurcation above the knee. The specimen was fixed in absolute alcohol and stained with iron haematoxylin. The two bundles are separated by a strand of muscle. x 130.

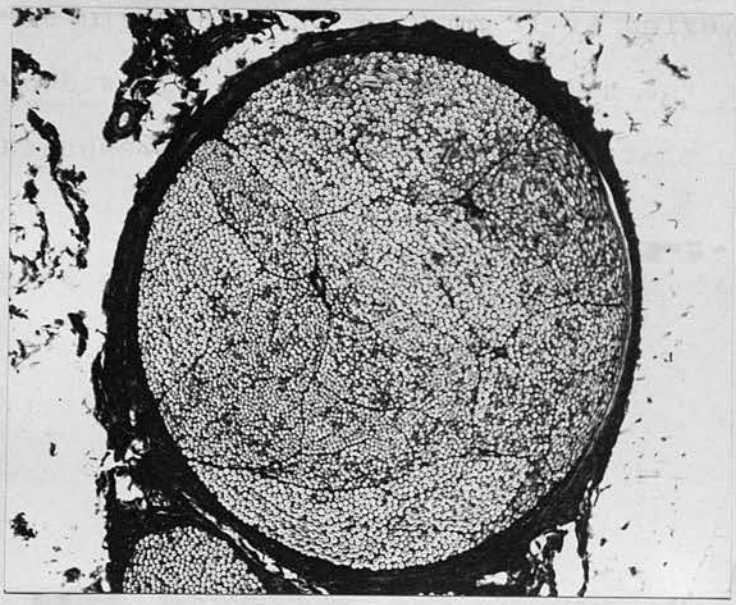

Fig.28. Transverse section of a sciatic nerve of a cat fixed in absolute alcohol, and stained with iron haematoxylin. x 50.

than the lower half (the peroneal nerve and its branches) no doubt because of the greater number of vessels involved. To find whether the avascular patches occur in a similar order in different nerves, the counts of the upper halves of 3 nerves were plotted on one graph (fig.26). Since the nerves had not all been cut at exactly similar levels, the curves were compared by taking the region of the sciatic bifurcation as a common point of reference. It can be seen that there are two principal peaks which correspond in the 3 nerves. The first peak, in the region of 12 mm., is at the origin of the nerve to the hamstrings. It is followed by a relative plateau where there are no pronounced changes in vascularity. The second peak, in the region of 30 mm., is at the main sciatic bifurcation above the knee. Both peaks are preceded by a stretch of relatively avascular tissue, which is evident as a very definite depression in each curve.

Morphology of the connective tissue in the frog sciatic nerve.

Histological studies of frog sciatic nerves have shown certain features which do not agree in some respects with the standard descriptions, based largely on studies of large mammalian nerves.

1. The connective tissue of a frog nerve is much less conspicuous than that of a typical mammalian nerve (fig. 27 and fig. 28).

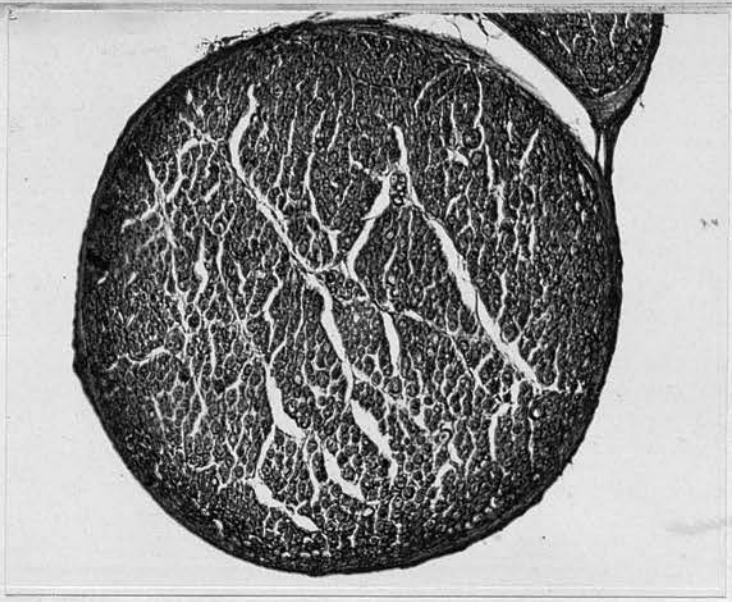

Fig.29. Transverse section of a frog sciatic nerve treated with strong acetic acid and stained by Mallory's technique. Long strands of swollen collagenous tissue can be seen here and there, providing a rather vague framework for the nerve fibres. x 135.

Fig.30. Detail from fig.28 showing an epineural connection between the two bundles. x 600.

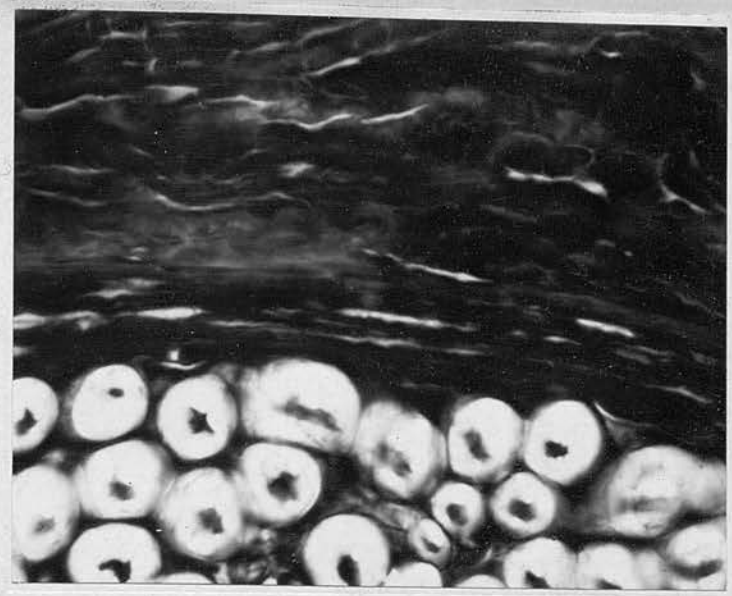

Fig.31. Detail from fig.27, showing the lamellated structure of the perineurium of a typical mammalian nerve. The abundant but less regularly arranged tissue of the epineurium is also seen. x 650.

Fig.32. Part of transverse section of a frog sciatic nerve after an intraneural injection of 10% silver nitrate, and staining with Ranvier's picrocarmine. The two dark lines in the perineurium (seen especially well where it has separated a little from the bundle) mark the two endothelial cell layers. x 700.

2. The epineurium is usually no more than loose adventitial tissue. When two nerve bundles are present, they may be attached by what is apparently a condensation of such tissue (fig. 29 and fig. 30). However, the common sheath seen where a nerve divides is really the perineurium, a layer of which extends gradually between the two incipient bundles and then splits to give an independent covering to each.

3. The perineurium is not a multi-lamellated structure, as in a large mammalian nerve (fig. 31). It apparently has two layers of endothelial cells which can be demonstrated with silver nitrate (fig.32). The intermediate layer of tissue is probably collagenous material, which is coloured pale blue by Mallory's technique.

4. The endoneurium does not show at all well in ordinary transverse section (fig.27). There are no obvious strands dividing the bundle into compartments with large vessels running within the strands as in a mammalian nerve (fig. 28). However, preliminary treatment with strong acetic acid causes swelling of the collagenous fibres, and an inchoate network of endoneural strands may then sometimes be distinguished (fig. 29). With a suitable silver impregnation technique, the endoneurium is seen more clearly (fig. 33). The relatively thick fibres of the fibrillenscheide show as black dots, distributed irregularly throughout the section. It is clear

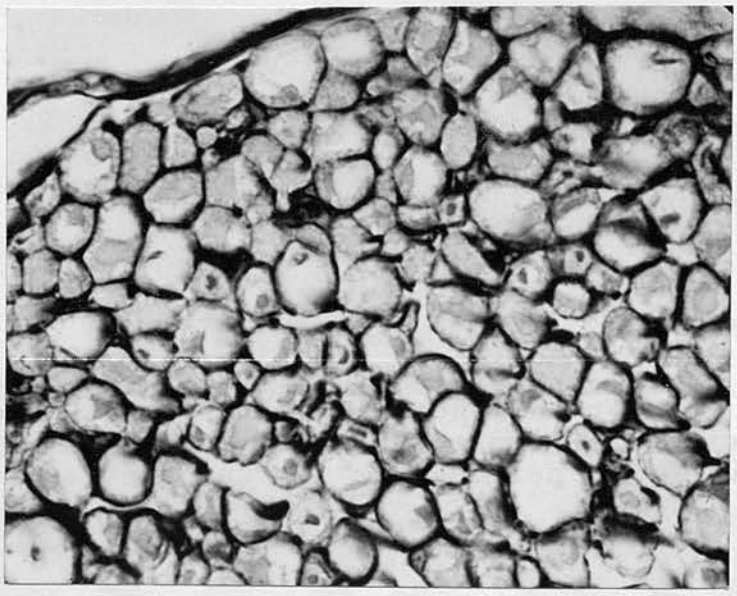

Fig.33. Part of transverse section of a frog sciatic nerve after silver impregnation by Laidlaw's method. The perineurium and the endoneurium are stained black. x 650.

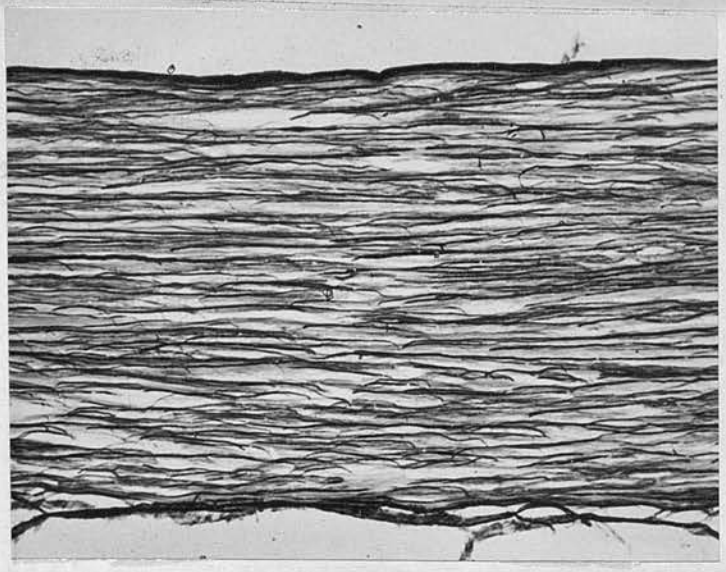

Fig.34. Longitudinal section of a frog sciatic nerve after silver impregnation by Laidlaw's method, showing the perineurium and the endoneurium. x 150.

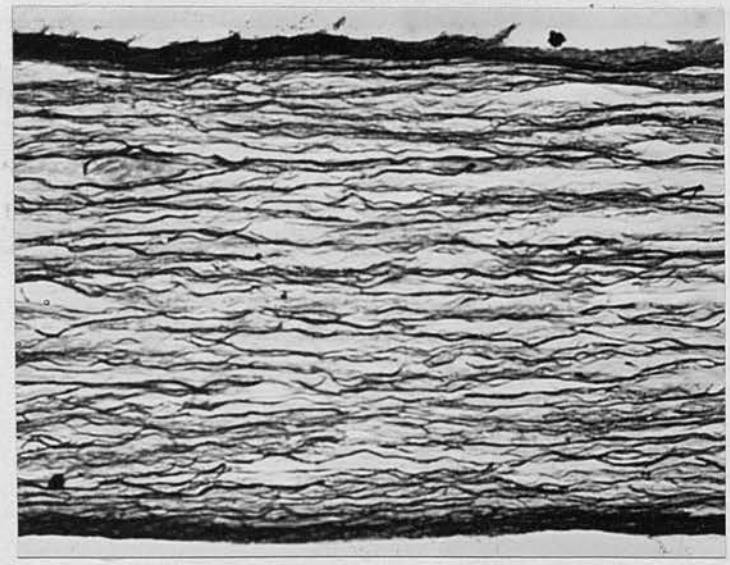

Fig.35. Longitudinal section of another frog sciatic nerve after silver impregnation by Laidlaw's method. x 250.

that they do not form a continuous envelope around the nerve fibres. Longitudinal sections impregnated with silver give an excellent picture of the fibrillenscheide (figs. 33, 34 and 35). The low-power views show that there is a good deal of connective tissue inside the nerve, as Lorente de No has claimed, but the contrast between the thick, continuous perineurium and the comparatively loose network of the fibrillenscheide is well brought out, especially in the high-power views (fig.35).

There is a close relation between the endo-neurium and the intraneural capillaries, which are separated at least partly from nerve fibres by the fibrillenscheide (fig. 36).

The Plenck-Laidlaw sheath in the frog sciatic nerve.

Longitudinal sections impregnated with silver by Laidlaw's method have revealed the presence of an extremely fine fibrillar network which apparently covers the individual axons (Figs. 37 and 38). This probably corresponds to the fibrillar system described by Plenck and Laidlaw in mammalian nerves.

The fibrils are only seen at all clearly at a very high magnification (with an oil immersion objective), and only where the section coincides with the surface of a nerve fibre. They are much finer than the fibres of the fibrillenscheide between which they lie, and they usually run diagonally over the axon in a more or less criss-cross pattern.

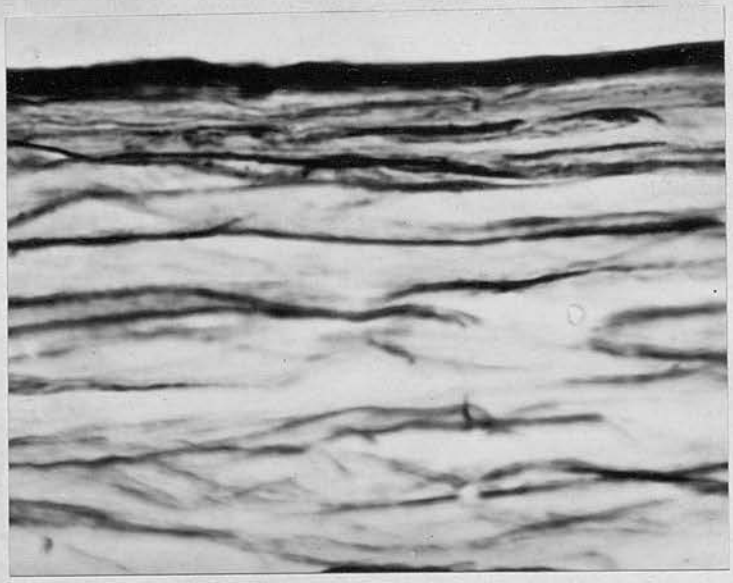

Fig.36. Detail from the section shown in fig.33 giving a closer view of the perineurium and the fibres of the fibrillenscheide. x 850.

Fig.37. Detail from a longitudinal section impregnated with silver by Laidlaw's method showing the fibrillenscheide in relation to an intraneural capillary. x 1200.

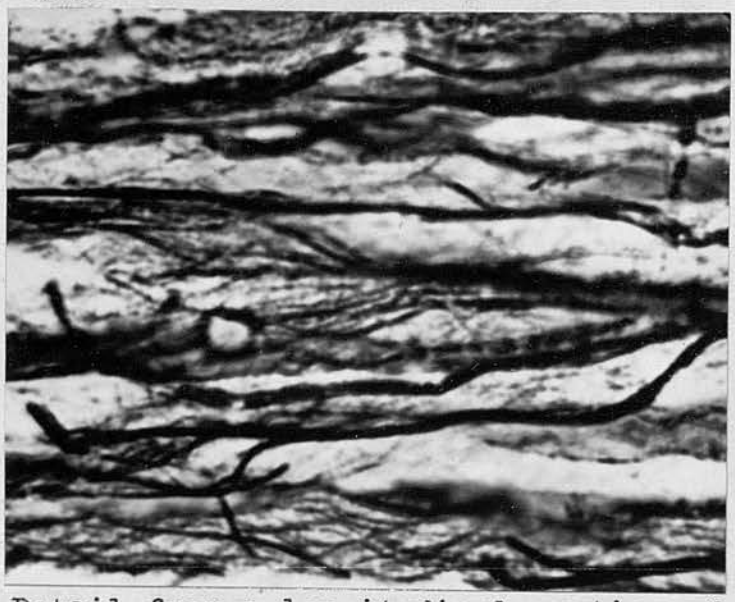

Fig.38. Detail from a longitudinal section of a frog sciatic nerve impregnated with silver by Laidlaw's method. A fine fibrillar network can be seen between the coarser fibres of the fibrillenscheide x 1500.

Fig.39. Detail from a longitudinal section of another nerve counterstained with ½% azocarmine after silver impregnation by Laidlaw's method. Fine fibrils form a criss-cross pattern over the axon which was stained by the azocarmine. x 1500.

Sometimes they seem to branch off fibres of the fibrillenscheide, as though it was all one continuous system.

Morphology of desheathed frog sciatic nerves.

The process of desheathing is always accompanied by a change in the appearance of the nerve, even when seen with the naked eye. This is not just a change in the surface texture, as the bright sheath is removed. There is always some loosening of the structure as a whole; the nerve tends to become flatter and the fibres to separate.

When histological sections of such a nerve are examined, the naked eye impression is confirmed. Instead of a compact nerve, with closely packed fibres surrounded by a distinct perineurium, one finds the fibres in a loosened bundle, the outline of which lacks the regularity and the smooth line of a perineurium (fig. 40 and 41). Even such a "good" specimen as fig. 40 shows an increased separation of the fibres throughout the nerve as well as on the surface. The structure of the nerve presented in fig. 41 is considerably loosened, and the general flattening is obvious. The uneveness of the section is at least partly due to the irregular alignment of the fibres. Yet this nerve, which might seem in a grossly abnormal condition, gave a satisfactory action potential.

The degree of change which is seen after

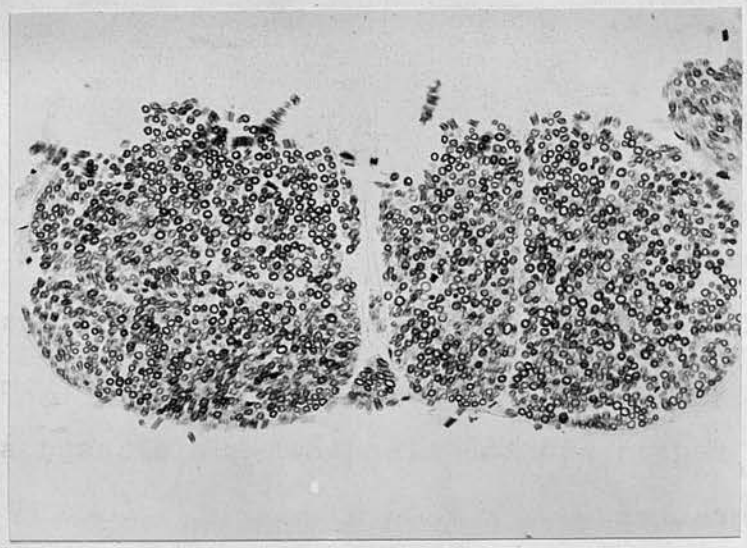

Fig.40. Transverse section of pelvic portion of a desheathed frog sciatic nerve fixed in Fleming's solution. x 75.

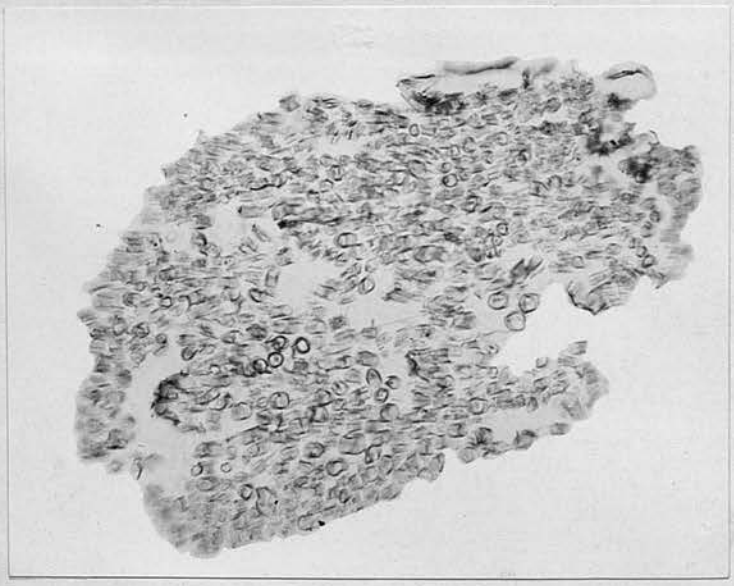

Fig.41. Transverse section of a desheathed frog peroneal nerve, fixed in Fleming's solution. x 185.

desheathing depends upon such factors as the care taken in dissecting the sheath, the thickness of the nerve, the distance of the site observed from an intact region and the time that has elapsed since the operation.

Table 3. Summary of results of perfusion experiments.

Blocking times (minutes).

Solution	Non-perfused (N.P)	Perfused (P)	Approx. ratio $\frac{NP}{P}$
Na free	180-360	6-7	30-60
0.12M KCl	25	1-1½	c.20
0.10M RbCl	36	6	6
Acid Ringer	40	6	6.6
0.10M $BaCl_2$	120	8	15
0.10M $HgCl_2$	22	¾	29
0.10M $CuCl_2$	75	6	13
0.90M $CaCl_2$			
i) without 0.011M NaCl	420	9	47
ii) with 0.011M NaCl	300-360	No block after 120	-
0.10M NH_4Cl	50-65	40	1.2-1.6
10% v/v acetone	2	2	1
10% v/v ethyl alchohol	6-7	8-9	0.75
0.015M cocaine HCl	3-11	12-25	0.5
1/5000 veratrine HCl	22	8	2.7

DISCUSSION

The experimental results have shown clearly that many solutions which interfere with the ability of the frog sciatic nerve to conduct impulses do so much more rapidly when they are introduced into the vascular system of the nerve than when they are simply allowed to act upon the whole, intact nerve. These are: Na free solutions, isotonic solutions of KCl, RbCl, acid Ringer (pH3), $BaCl_2$, $HgCl_2$, $CuCl_2$. On the other hand, solutions of certain substances, which are known to be lipoid soluble, act nearly equally rapidly in the two cases, while a third group which includes isotonic $CaCl_2$ and NH_4Cl gives rather ambiguous results.

It is well known that experiments in which intact and desheathed frog nerves are compared give results very similar to these, (Feng and Liu, 1949a; 1950; etc.). An explanation was offered by Feng and Liu (1949a) for each of the three types of result. They postulated that the total time taken by any substance to produce a certain observable effect upon a nerve is the sum of an entrance time, the time required for the substance to reach the nerve fibres, and an action time, the interval of time during which it must act before the effect appears. Those substances to which the nerve sheath is a barrier will have long entrance times; if their action time is short, removal of the sheath will make an

appreciable difference to the total time (group 1.) but this will not be so if their action time is long (group 3.). The second group of substances readily penetrate the nerve sheath and so the total time is only dependent upon the action time.

A perfused nerve can be considered as a system in which the sheath is bypassed and therefore equivalent to a desheathed nerve. However, one important difference is obvious: if it is assumed that the main process involved is diffusion within the nerve, then the two systems cannot be compared unless their effective dimensions are similar. But it was shown that the frog sciatic nerve is supplied with some 50 capillaries/mm^2. Diffusion times must be very different in the two cases since they vary with the square of the distance. It is therefore unjustified to compare the results of desheathing and perfusion experiments directly; the only comparison which can be made is of the respective diffusion constants calculated from results obtained in experiments with intact, desheathed and perfused nerves.

It is reasonable to consider the whole problem as one of diffusion. Rashbass and Rushton (1945) suggested that the nerve sheath may act by preventing mixing; they illustrated their argument by referring to diffusion and mixing of large volumes of $CuSO_4$ and Na_2SO_4, which is a little misleading since

penetration by diffusion in a small system such as the frog nerve is extremely rapid (Hill,1928). In a good desheathed preparation, mixing probably plays only a small part as shown by Feng and Liu (1949,a) with desheathed nerves coated with gelatin. The separation of fibres in such nerves is apparently caused by swelling of the endoneurium (Lorente de No, 1952d). However, mixing probably accounts for very short blocking times in some preparations.

Diffusion measurements are sometimes distorted by filtration (Jacobs, 1935; Ussing 1949), the movement of a solution as a continuous phase resulting from differences in hydrostatic and osmotic pressures. This can only take place through a membrane if sizeable pores are present which allow both solvent and solute particles to pass. The ability of the frog perineurium to prevent the outward passage of solution of methylene blue at even high pressure suggests that such pores, if at all present, must be rather fine. Further, the rigidity of the sheath tends to keep the volume of the nerve constant so that significant inward movement of water can only take place after desheathing (Lorente de No, 1952d). Excessive filtration through the capillary pores during perfusion is equally unlikely. Pappenheimer et al (1952) have shown that under normal conditions of hydrostatic and osmotic pressure in the perfused hind limb of the cat, the exchange of NaCl by diffusion is about 260 times greater than by filtration.

Of the various results described earlier only

two groups, those dealing with Na free and with KCl solutions, lend themselves to an exact mathematical analysis. It is only in these two cases that there is good evidence for correlating conduction block with a definite local concentration of the diffusing substances. An attempt at evaluating a coefficient of diffusion in the nerve will, therefore, only be made for Na and for K.

Equations which can be used for the study of the kinetics of diffusion in intact and desheathed nerves were described by Hill (1928). For the case of the perfused nerve, the conditions are somewhat more complex. If, however, the nerve is considered as an aggregation of smaller cylinders, each having a capillary at its centre, then it is possible to deal with it by the type of equation described by Muskat (1937, p. 654) and Roughton (1952, 3.10,p.207) for a system of two concentric cylinders. The approximation is that made by Krogh (1919b) when calculating the rate of diffusion of O_2 in muscle; with a nerve, which has a smaller concentration of capillaries, the approximation is, no doubt, a little less exact but the capillaries are sufficiently uniformly distributed to give an answer of the correct order of magnitude. It willbe noticed that this treatment requires that no diffusion takes place through the outer boundary of each system of concentric cylinders. This can be assumed to be

true, especially as far as the innermost cylinder is concerned, as long as the total diffusion times are much shorter than in the non-perfused nerve.

Since the various fibre types are distributed evenly throughout the nerve trunk, it can be assumed that the onset of complete inexcitability is associated with the attainment of threshold concentration at the axis of non-perfused nerves. In the perfused nerve, the corresponding time theoretically will be the attainment of threshold concentration at the boundary of the innermost system of cylinders, which is least likely to be affected by external diffusion.

One difficulty must also be considered. Although both Na and K have been shown to penetrate the axon membrane, in effect, the axon behaves as an impermeable system, so that the nerve fibres with their lipoid myelin sheaths, cannot be included in the present scheme of diffusion, What is the effect of these non-conducting bodies, which, as a glance at the cross-section of any nerve will show, occupy a major part of the total area? It has been assumed sometimes that the nerve fibres must act as obstructions which prevent diffusion, and it has been suggested that the slow diffusion of electrolytes in intact nerves is at least partly due to this factor. Shanes, in the discussion printed after Lorente de No (1952d) commented that the

effective diffusion coefficient in a nerve is reduced in proportion to the distance between the fibres relative to their diameters. This is probably not the case. If the problem is simplified a little by considering the fibres as small non-conducting squares, instead of cylinders, distributed uniformly, it can easily be shown that the real value of D can be obtained by multiplying the apparent value by $\frac{1-Z}{1-\sqrt{Z}}$, or $(1+\sqrt{Z})$ where Z is the relative area of a non-conducting square in terms of unit total area. The value of $1+\sqrt{Z}$ tends towards 2 as Z approaches 1. The fibres in a frog nerve are by no means perfect cylinders (fig. 33) so that the simplification is not unreasonable. It is therefore not likely that the conspicuously slow diffusion of electrolytes in intact nerves can be caused to a great extent by the presence of the impermeable nerve fibres.

what solid?

D not defined

<u>The diffusion constant of K and Na in the frog nerve.</u>

1. Intact nerve in 0.12M KCl solution:

The corresponding equation is given by Hill (1928, p.70, no.46); he later (1948) altered it by changing the minus sign before the square bracket to plus, but this cannot be correct since the right hand side of the equation can never exceed 1. This ambiguity must be ascribed to a series of misprints on p.71 (1928) which give the wrong sign before values of $J_1(\gamma_n)$.

$$\frac{c}{C} = 1 - 2 \sum_{n=1}^{\infty} \frac{e^{-\frac{D\beta_n^2 t}{a^2}}}{\beta_n J_1(\beta_n)} \qquad (1)$$

where a = radius of nerve

c = concentration at the axis

C = concentration maintained constant outside.

β_n = the zeros of $J_0(x)$

Lorente de No (1947a) has claimed that even 11mM KCl can produce conduction block in a frog nerve, while Huxley and Stämpfli (1951) stated that action potentials of single nerve fibres were not seen when the external K concentration was 20mM or more. It was therefore assumed that total block occurred at c = 20mM, neglecting the initial small normal concentration of K. With a = 0.027 cm (from direct measurements on sections of nerves) and t = 25 min., the corresponding value of D is 3.5×10^{-6} $cm^2/min.$

2. Intact nerve in Na free solution:

In this case, Na diffuses from the nerve into the surrounding solution in which its concentration is maintained at 0. The equation is derived from (1) by subtraction.

$$\frac{c}{C} = 2 \sum_{n=1}^{\infty} \frac{e^{-\frac{\beta_n^2 Dt}{a^2}}}{\beta_n J_1(\beta_n)} \qquad (2)$$

In this case, C is the original concentration of Na, and it is assumed that complete block occurs at $c = \frac{1}{10} C$ (Overton, 1902b; Lorente de No, 1947; Huxley and Stämpfli, 1951; etc.) when a = 0.027 cm., and t = 300 min., D = 1.2×10^{-6} $cm^2/min.$

3. Desheathed nerve in Na free solution:

The same equation is used but with t = 5 min., when D = 7.2×10^{-5} $cm^2/min.$

4. Nerve perfused with 0.12M KCl:

The equation for a suitable system of concentric cylinders given by Muskat (1937) p.654) is a little simpler than that of Roughton (1952, 3.10, p.207), but as it only holds strictly for values of the order of 2,000 or more, the latter is preferable.

$$c_a = c_b - \pi(c_0 - c_b) \sum_{n=1}^{\infty} \frac{J_0(x_n)\, Y_0(x_n\theta) - J_0(x_n\theta) Y_0(x_n)}{\left[\frac{J_0(x_n)}{J_1(x_n\theta)}\right]^2 - 1} e^{-\frac{x^2_n Dt}{b^2}} \qquad (3$$

where a = radius of larger cylinder

b = radius of smaller inner cylinder

and $\frac{a}{b} = \theta$, C_o is the initial concentration in the nerve.

x_n are the positive roots of

$$U_{(x_1\theta)} = J_0(x)Y_1(\theta x) - Y_0(x)J_1(\theta x) = 0$$

which can be obtained either from a graph or by using a table computed by Roughton (1952, p.209). It was assumed that the concentration was maintained constant at b for all values of t 0; and that block occurs at c_a = 20 min. The average highest concentration of vessels in sciatic nerves ($68/mm^2$) (S.D. ≈ 12/mm²) correspond to $a = 70 \times 10^{-4}$cm, while b was taken as 5×10^{-4}cm. (i.e. the radius of the average capillary). The value of D which corresponded to t = 1 min., was $1.4 \times 10^{-5} cm^2/min$.

5. Nerve perfused with Na free solutions:

The equation is again (3), but with suitable changes in the values of c_a and c_0, and it was now assumed that block occurs at $c_a = \frac{1}{10} C$. When t = 7 min. the value of D is $1.61 \times 10^{-5} cm^2/min$.

The values of D (coefficient of diffusion) obtained in the five cases were:

For Na:

Intact nerve	1.2×10^{-6} $cm^2/min.$
Perfused nerve	1.6×10^{-5} $cm^2/min.$
Desheathed nerve	7.2×10^{-5} $cm^2/min.$

For K:

Intact nerve	3.5×10^{-6} $cm^2/min.$
Perfused nerve	1.4×10^{-5} $cm^2/min.$

D for 0.1M solutions in water at 18°C is given in the International Critical Tables as:

7.8×10^{-4} $cm^2/min.$ for NaCl.

9.1×10^{-4} $cm^2/min.$ for KCl.

The values of D in intact nerves were 1/650 (Na) and 1/260 (K) of the value in pure water. These figures are certainly of the right order of magnitude; if a correction is made for the presence of impermeable nerve fibres, the coefficients will not be much more than twice these values. D has a temperature coefficient (Q 10) of about 1.3 which would tend to counteract the increase because most of the experiments were done at temperatures in the region of 20°C.

The relatively high value of D for K is interesting. It is not decreased at all significantly by assuming that conduction block occurs even at 10mM. It is perhaps not unjustified to suspect that the difference is a genuine reflection of the effect-

iveness of the perineurium as a barrier to the diffusion of the two ions respectively.

The conclusion that only the impermeability of the perineurium can account for such low values of D is supported strongly by the high value of D for Na calculated for desheathed nerves. 7.2×10^{-5} $cm^2/min.$ is only about 1/10 D for water, and it may be even greater than that if an allowance is made for the fibres. According to Shanes, (Lorente de No, 1952d) the weight of a desheathed bullfrog nerve increases by 20% in 10 minutes, but further change is slow. Filtration is therefore not likely to play an important part by the time a nerve is placed in the Na free solution. On the other hand, some loosening of the structure of the nerve will increase the effect of mixing and so tend to increase the apparent rate of diffusion.

One may note at this point that Fenn (1928), calculated the value of D for CO_2 in intact nerves to be 7.1×10^{-5} $cm^2/min.$ The values of D for CO_2 and K (in water) are very similar, but according to Fenn, the value of D for CO_2 in muscle is nearly 1.8 times greater than that for nerve. It seems therefore, that CO_2 does not diffuse in nerves quite as rapidly as its lipoid solubility might suggest.

In the case of both Na and K, the value of D in the perfused nerves was substantially higher than in the intact, nonperfused nerves, (13 and 4 times respectively). It is not surprising that the

difference is not greater. The number of assumptions and approximations made in estimating D was greater than in the previous cases, and all these assumptions were such as to weigh against a high rather than a low value of D. It is not likely that the concentrations in the capillaries was really maintained at 0 or 0.12M KCl from t = 0 with Na free and KCl solutions. The dead space in the system can be ignored usually but in the case of 0.12 M KCl, the total blocking time was of the order of 1 minute only, and so a delay of even only ½ minute will double the calculated value of D.

When considering diffusion in the case of the concentric cylinders, it has been assumed so far that the inner boundary of the system was freely permeable to electrolytes. But there is some reason to believe that the capillary wall is not equally permeable in all parts. Chambers and Zweifach (1947) suggested that the intercellular cement of the capillary, which occupies only 1% of the total surface area, is the principal pathway for diffusion. This was contested by several observers (e.g. Flexner et al. 1952; Cowie et al., 1949) but Pappenheimer et al., 1952, have claimed that only 0.2% of the capillary wall in cat muscles is available for diffusion or filtration, although they were unable to decide whether the permeable pores are uniformly distributed over the endothelial cells or concentrated in

specific regions. It is evident that the nerve capillary may also be only partially permeable, and this may explain the comparatively low value of D in perfused nerves, as well as some of the difficulties experienced with gelatin perfusates, which probably tend to obstruct any pores present.

It seems, however, that diffusion into, or from, perfused capillaries should be considerably slower if only 0.2% of the wall is permeable. The present results do not support Pappenheimer's claim quantitatively since although they were calculated on the assumption that the capillary wall is 100% permeable, the value of D for the perfused case was ~~only~~ 1/5 - 1/4 that for the desheathed. (The similarity between the values of D for Na and K suggests that the relative impermeability, in contrast to that of the sheath, is non-selective in this respect.)

The very high values of diffusion coefficients of Na calculated previously by Lorente de No (e.g. 1952d) were based upon very arbitrary assumptions. It is well known that the nerve is exceedingly sensitive to very small variations in the concentration of Na near the conduction threshold; the very rapid response of intact nerves to Na under these conditions and the spontaneous recovery seen in air and other similar phenomena, caused either by drying of the nerve or diffusion of Na from fibres or

connective tissue, need no elaborate explanation. The other diffusion studies by the same author of lipoid soluble substances (cocaine and procaine) are not relevant. Such substances are known to penetrate cells without difficulty; and the relatively high values of D in intact nerves estimated for O_2 (Gerard, 1927) and CO_2 (Fenn, 1928) follow from their high oil-water partition coefficient. Cocaine at a physiological pH is in the form of the amine (Woods et al., 1951a) which has a very high affinity for lipoid tissue. It seems probable that veratrine is also able to penetrate cellular membranes, even when administered as a salt, although there is little direct evidence on this point.

Before leaving the subject of the perfusion experiments, a comment is necessary on a few other features.

1. The contrast between the results of perfusion with strong $CaCl_2$ in the presence and absence of 0.011M NaCl suggests that Feng and Liu (1949a) failed to take the precaution of retaining a sufficient amount of Na when they found that strong $CaCl_2$ produced rapid block.

2. The enhancement of recovery after block by acetone and ethyl alcohol in perfused nerves shows that the diffusion time is substantially shorter than in non-perfused nerves. The quick diffusion seen here supports the hypothesis that it is a cellular

barrier which slows down the movement of electrolytes from or into the capillaries.

3. The early loss of excitability of frog muscles perfused with Na free solutions can be ascribed to their richer supply of vessels.

4. The effect of the vasodilator and vasoconstrictor drugs upon the total fluid content of the nerve is an example of the importance of its vascular supply. The tone of the arterioles produce changes in the longitudinal impedance which must be even greater than those measured externally. One may speculate on the possibility that changes in the external resistance of the axon might be great enough to affect the conduction velocity.

5. The very rapid action of cocaine on desheathed nerves described by Feng and Liu (1949, a; 1950) is not seen in perfused nerves. It is possible that desheathing does sensitize the fibres to the action of cocaine.

6. The low $\frac{N.P.}{P}$ ratio for Rb is surprising. It may be that a few minutes are required for the full action of Rb at the blocking concentration, or that the capillary is relatively impermeable to it. It is likely that the comparatively slow onset of block with perfused acid Ringer is the result of a progressive, rather than an instantaneous action on the nerve fibre.

7. Whatever the view taken of the properties of

the nerve sheath, it cannot be denied that the rapid loss of excitability of intact nerves perfused with isotonic Na free solutions gives strong support to Hodgkin's theory of nerve conduction. There is no foundation for Lorente de No's (1952d) suggestion that Na free solutions act differently in perfused as well as in desheathed nerves.

The blood supply of the frog sciatic nerve.

The uneven distribution of vessels is reflected in the high values of the standard deviation of counts. It is not yet clear to what extent technical deficiencies are responsible but it has been shown that a recognisable pattern of vascularity can be discerned in different nerves associating high concentrations of vessels with important bifurcations and with close proximity of the companion artery.

The most important result of these studies is undoubtedly the demonstration of a vascular supply whose average concentration is of the order of 50 vessels/mm^2. About 90% of these are certainly capillaries, while the remainder, with diameters greater than some 14μ, may be arterioles and venules, (Zweifach, 1937).

According to Krogh (1919a; 1922) the corresponding concentration of capillaries in frog muscles is 300-500/mm^2 but he also quotes (1919c) counts of 10 and 90 in resting muscle. Even the higher figures are not as great in comparison as might be expected

from the relative requirements of the two tissues in O_2. The dimensions of a frog nerve are such that its resting O_2 needs are supplied adequately by only 1% external O_2 (Gerard, 1927). If there were free diffusion into and out of the nerve, a blood supply would be almost totally unnecessary, but this is evidently not the case. The rich blood supply of the peripheral nerve trunk, may be regarded as a corollary of the impermeability of the perineurium. Calculations of the vascular volume of the nerve give a value of about 0.05% compared with 5-10% for frog muscle estimated by a rather indirect method (Danielli, 1941; Danielli and Davson, 1941).

Although there are good descriptions of the blood supply of nerves in the literature (Ranvier 1878; Adams, 1942; Sunderland, 1945) no quantitative studies have been found with which the present results might be compared.

The perineurium and the diffusion barrier.

The lamellated sheath of the frog nerve is the most substantial element of its connective tissue. Lorente de No's stress of the importance of the endoneurium does not bear full examination. Even if the total amount of the latter tissue does exceed that of the former, it is surely evident that the arrangement and the nature of the endoneurium prevents it from acting as a barrier. The perineurium, on the other hand is an unbroken cellular

membrane (see Key and Retzius, 1876, fig.10 plate 15) which covers the whole trunk. It probably consists of only one lamella, covered on both sides by endothelial cells forming a double cellular layer which accounts for its apparent impermeability. The operation of desheathing involves removal of the perineurium; it is always accompanied by a great increase in the rate of diffusion of electrolytes if at all successful. The transparent and extremely thin structure of the perineurium makes its removal rather difficult. The mistaken conclusion reached by Causey and Palmer (1953) that it is the epineurium which is the diffusion barrier is based, judging from their illustrations, upon the histological examination of "desheathed" frog nerves whose [1]amellated sheath had remained in position.

It can now be concluded that the short time constant of diffusion in perfused, intact nerves, the inability of a solution of methylene blue injected intraneurally to escape to the surface unless enormous pressures are employed, together with such indirect evidence as the histological structure and the rich blood supply of nerves, give substantial confirmation to the widely held belief that the frog nerve sheath is a diffusion barrier. The barrier is probably situated in the endothelial cell layers of the perineurium. Its permeability to water may depend upon the presence of pores (cf. capillaries)

which are too fine to allow the passage of all but the smallest particles. The appreciable difference between the diffusion constants of Na and K in intact nerves suggests that the dimensions of the hypothetical proes may be comparable to those of the K ion in solution.

The perineurium (lamellated sheath) in relation to the cerebrospinal meninges.

It may seem surprising a priori that the peripheral nerve trunk should be surrounded by a relatively impermeable sheath. One may well expect to find connective tissue supporting and holding together the nerve fibres but why should a portion of it be a diffusion barrier ? Why should this lamellated sheath which is usually said to be mesodermal in origin constitute such a barrier around the ectodermal nerve fibres separating them and the mesodermal endoneurium from the surrounding tissues from which it is itself derived ? Since such a question can hardly be answered directly, it may be profitable to examine other parts of the animal organism with the hope that a comparable system of connective tissue sheaths may give some clue to the nature and the functions of the perineurium.

One need not go at all far to find a remarkably close parallel in the meninges of the central nervous system. This is all the more satisfying since there is no reason to believe that the properties of the central and peripheral nervous tissues in every respect differ fundamentally. Therefore to compare the meninges and the peripheral sheaths is not likely to be grossly misleading.

It will be remembered that the meninges consist of one or two external layers of dura mater (the pachymeninx) and of two internal layers of leptomeninx,

the relatively loosely fitting arachnoid mater, and the vascular pia mater which is closely applied to the nervous tissue. The arachnoid mater is of particular interest because it encloses the subarachnoid space, and therefore an important part of the cerebrospinal fluid, sometimes known as the "extracellular fluid" of the central nervous system. The arachnoid mater resembles the peripheral lamellated sheath in that it possesses a matrix of fibrils, covered on both sides by flattened endothelial cells, and accompanies all blood vessels passing to the brain or spinal cord (Weed, 1938). The subarachnoid space extends within the nervous tissues in perivascular cuffs, by which it may be continuous with the extracellular spaces.

This standard description of the meninges may not apply in every respect to these membranes as found in the frog. The principal difference is said to lie in the presence of an apparently undifferentiated leptomeninx consisting of only one membrane, and in a number of extensions of the endolymphatic system of the inner ear, including one in the spinal canal where it is found within the two layers of dura, as the Saccus Endolymphaticus filled with a fluid rich in Ca (Ecker and Weidersheim, 1899: Sterzi, 1902.) However, a study of the meninges in Amblystoma Punctatum by Flexner (1929)) suggests that the three definitive membranes develop in amphibians essentially as in mammals.

The evident morphological resemblance between the arachnoid mater and the perineural sheath suggests that there may be a closer connection between these structures than is apparent in the standard textbook picture, according to which the only feature the peripheral sheaths and the meninges have in common is a mesodermal origin. There has been found in the literature powerful evidence that the leptomeninx and the perineurium are homologuous structures, both derived from neural crest ectoderm.

Harvey and Burr (1926) have shown that the leptomeninx is developed from migratory cells of the neural crest, a cord of ectodermal tissue lying in the angle between the neural tube and the surface ectoderm, which is known to give rise to the spinal ganglia. They demonstrated in amphibian embryos the failure of brain transplants to develop a leptomeninx when neural crest cells were absent, and described in detail the growth of the leptomeninx and pachymeninx in mammalian embryos. According to these authors, the dura mater is probably largely derived from the mesoderm. Flexner (1929) disputed some of these conclusions, but his own experiments did not lead to a fundamentally different interpretation; Harvey et al. (1933) then gave more evidence in support of their original thesis, but agreed that the pachymeninx in the head region may contain some ectodermal elements. The fairest estimation of the position is perhaps that

of Leary and Edwards (1933) who compared in some detail the histological structure of the dura, the arachnoid and serous membranes such as the pleura, and came to the conclusion that these three membranes should be distinguished sharply, and that the dura is predominantly mesodermal while the arachnoid is predominantly ectodermal.

It has been known since Harrison's work (1924) that amphibian neural crest cells migrate out with the peripheral nerves in which they ultimately constitute the neurilema. Only comparatively recently, however, has Masson (1942) shown by a careful study of human embryos that these neural crest cells ("primitive Schwannoglia") are the precursors of all the connective tissue elements of the peripheral nerve, bar the epineurium. He described the gradual differentiation of the schwannoglia into the various elements of the perineurium and endoneurium, and noted that blood vessels only grow into the nerve when there is present a well defined endoneural network. According to Masson, these blood vessels and their adventitial cells are the only tissue of mesodermic origin to be found in nerves. Hence there are no lymphatics present, and lymph must therefore circulate in endoneural and perineural spaces before diffusing out. Masson's studies of encapsulated tumours of the peripheral nerves demonstrated their close resemblance to other neural crest cell tumours

of the meninges.

Whether there is anatomical continuity between the various meninges and peripheral sheaths is a matter of dispute. Tarlov (1937), who has made an intensive study of the junction between the central and peripheral nervous tissues, stated that the dura, arachnoid and pia are continuous with the epi-, peri- and endoneurium respectively. Hassin (1947) supported this view, but Somberg (1947) and Brierley (1950) contested its truth without, however, their own descriptions agreeing. It seems that the arrangement of the sheaths in relation to the roots and spinal ganglia does not lend itself to a very simple description, and that no definitive statement can be made as yet. This is perhaps not altogether surprising if it is remembered that the spinal ganglia, the leptomeninx, the peripheral neurilema, the perineurium and the endoneurium are all derived from the same neural crest cells.

A problem of great physiological importance is closely related to the above. Are the peripheral perineural and endoneural spaces directly continuous with the central subarachnoid space ? This question has received a great deal of attention in the past in several fields of applied physiology, in bacteriology and pathology, particularly with respect to the study of the spread of tetanus toxin and various neurotropic viruses. No attempt will be made to

review here fully all the experimental work which has been done, but a summary of the results is justified by the importance of the subject.

Meyer and Ransom put forward in 1903 a comprehensive hypothesis of the mechanism of the spread of tetanus toxin based on clinical and experimental evidence. They claimed that the toxin elaborated by the infecting organism in muscles travels within the motor axons in nerve trunks to the central nervous system, where it then exerts all its actions. The hypothesis of neural spread has survived many assaults remarkably well; a series of papers by Abel and his associates (see Abel et al., 1938) supported the hypothesis of blood spread and denied the possibility of neural spread, but there has been strong support more recently from Friedemann et al. (1941) and Hutter (1949).

One of the main and commonest objections voiced against the neural spread hypothesis is that it is difficult to understand how the tetanus toxin can reach the central nervous system so rapidly by diffusion within the axons, especially since there is evidence that the toxin when it reaches neural tissue becomes firmly bound to it. Thus, Abel et al. (1938) state; "it would probably require several months for the assumed neural transportations of freely movable toxin by molecular forces by way of the sciatic nerve from an infected hoof of a horse to its spinal motorneurons". Hence many experiments have been

performed to find evidence for an alternative pathway within the nerves, with a view especially to finding a connection between the peripheral nerve "lymph spaces" and the subarachnoid space.

Key and Retzius (1876) had claimed that gelatin solutions injected under "low pressure into the subarachnoid space spread into the perineural spaces, and vice versa, but Weed (1914) using a controlled physiological pressure, could not trace injected fluid beyond the region of the spinal roots, where a certain amount of drainage out into the lymphatic and venous system apparently takes place. The importance of using controlled physiological pressures was stressed by Weed, who claimed that Key and Retzius' results were experimental artefacts easily reproduced with high pressures. A number of workers subsequently confirmed the absence of perineural spread from the subarachnoid space (Elman 1923; Iwanow and Romodanowski, 1928; Sullivan and Mortensen, 1934; Somberg, 1947; Brierley and Field, 1948) but others published evidence (Ponomareff, 1927; Wischnewski, 1928) which suggested that there is a centripetal flow of fluid within the interspaces of the nerve, and that the time taken for a substance to reach the central nervous system may be varied by raising or lowering the cerebrospinal fluid pressure. According to Iwanow (1928) fluid flows down from the upper regions, and up from the nerves, towards the

spinal roots, where the presence of outward drainage first noticed by Weed was confirmed by Elman (1923) and Iwanow (1928), and later by Iwanow (1929), Brierley and Field (1945), Howarth and Cooper (1949). It is difficult to know exactly to what extent that particular scheme of circulation depends upon the conditions of the experiment (e.g. raised pressure) and at least one feature was not confirmed by Eichler et al. (1951), who found, by means of tracer experiments, an ascending flow of cerebrospinal fluid in the spinal subarachnoid space. Elman (1923) described groups of cells of the arachnoid, near the spinal roots, which he claimed resembled the arachnoid granulations of the cranial sinuses. The greater permeability of the arachnoid in this region may be a property of these cells and it may be due to this that fluids injected into the subarachnoid space usually fail to pass out into the peripheral nerves unless high pressures are employed. Nevertheless, it is possible under physiological conditions for substances to travel into the nerves from the cerebrospinal fluid if sufficient time is allowed and their concentration in the cerebrospinal fluid is maintained at a high level. Brierley (1950) demonstrated the movement of Indian ink particles from the Cisterna Magna to the upper reaches of the sciatic nerve in some 48 hours; he thought that the subarachnoid space ends in a cul-de-sac near the

spinal ganglion, that the dura is continuous with the perineurium, and that the peripheral spread of particles occurs through the permeable arachnoid sac, and also directly into the spinal ganglion. Although Brierley's experimental evidence seems sound enough, his interpretation of the morphology of the parts cannot be accepted unequivocally since he makes no attempt to distinquish between the three peripheral sheaths.

The centripetal movement of various fluids injected into the peripheral nerve has been even more frequently the subject of experiments. A number of observers claimed that many fluids injected into nerves pass relatively easily and quickly into the subarachnoid space and the central nervous system (Key and Retzius, 1876; Teale and Embleton, 1919; Ponomareff, 1927; Wischnewsky, 1928; Yuien, 1928; Yuien and Sato, 1929; Horster and Whitman, 1931; Sullivan and Mortensen, 1934; Brierley and Field, 1949; Wright et al. 1950). The most spectacular evidence is given by Brierley and Field; 2 hours after injecting 0.05 cc. of a solution containing P32 into the sciatic nerve of a rabbit at the level of the popliteal fossa, they found a significant increase in the activity of the basal ganglia, some 35 - 40 cm. away. This remarkable result may well be a striking illustration of the importance of intraneural and neuro-subarachnoid

pathways. Unfortunately, although these authors employed control animals which received similar doses of P32 intravenously, they (like the other authors) failed to control the intraneural injection pressure, relying on the assumption that the injection of so small a volume of fluid cannot result in any great increase of pressure. Brierley and Field further assumed that the injection was quite localised, and that any spread found at death must be a true indication of normal, spontaneous lymph flow.

Both these assumptions are unjustified in view of evidence published previously: Mulder (1939) injected a great variety of fluids into rabbits' nerves in carefully controlled experiments. He stated that at a pressure of 40 cm. H_2O spread is very slow and occurs equally up and down, whereas an uncontrolled injection of even only 0.05 - 0.2 cc. leads to rapid centripetal filling of the nerve and its roots, and even of the spinal subarachnoid space.

It must be concluded that although there is much positive evidence, it would be premature to state categorically that there is a significant flow of extracellular fluid within the perineural spaces, and that a functional pathway exists between these and the subarachnoid space. It may well be that in this field very delicate equilibria of pressure exist, and that the normal function cannot be demonstrated unless exceptional care is taken to maintain the status quo and adequate time is allowed for the

completion of relatively slow but nonetheless essential processes (as in Brierley, 1950). The present experiments have shown clearly the inadequacy of uncontrolled injections and also of short term experiments with physiological pressures.

The function of the ectodermal sheaths of the nervous system.

It has been shown that the perineurium of the peripheral nerve is derived from the same embryonic tissue as the leptomeninx and that these two membranes jointly provide a probably complete ectodermal covering for the whole nervous system. Evidence has been cited which suggests that there may be direct continuity between the lymphatic or extra cellular fluid systems of the peripheral and central nervous tissues. To understand the properties and the function of the perineurium, it is now necessary to examine in some detail the regulation of the internal environment of the central nervous system in which the leptomeninx plays an important part.

The cerebrospinal fluid is produced mainly at the choroid plexuses inside the ventricles and re-absorbed into the blood of the cranial venous sinuses by filtration through the arachnoid granulations (Weed, 1914; 1938.) The process of formation of the cerebrospinal fluid has been a subject of some controversy but there is now overwhelming evidence that the fluid is not produced from blood by

ultrafiltration but that there is active and selective secretion by the cells of the choroid plexuses. Most of the evidence is based upon comparative analyses of the cerebrospinal fluid, blood serum and extracellular fluids, under normal and various experimental conditions, which show significant discrepancies between the respective concentrations of many substances, (Levinson, 1919; Flexner, 1934; Katzenelbogen, 1935; Friedemann, 1942; Greenberg et al. 1943; Tschirgi and Taylor, 1953). It has been found that some 13 calories are needed to produce 1 litre of cerebrospinal fluid, (Flexner, 1934) and that the choroid plexuses are the site of intense enzymatic activity (Leduc and Wislocki, 1952).

It has also been found that many substances act much more rapidly on the central nervous system when injected into the cerebrospinal fluid than when injected intravenously, (Stern and Gautier, 1922) and that several anions are distributed in the central nervous system in a ratio similar to that found in the cerebrospinal fluid, whereas in other parts of the body, the corresponding ratios are similar to those obtaining in blood serum, (Wallace and Brodie, 1938.) The penetration into the central nervous system of various substances injected into the blood stream is remarkably slow (e.g. Wislocki and King, 1936; Hiatt, 1939; Hahn and Hevesy, 1941; Manery and Bale, 1941; Friedemann

et al., 1941; von Rijssel, 1946; Bakay, 1951, 1952; Wislocki and Leduc, 1952). According to Wallace and Brodie (1940) it takes seven hours or more for Br, I or CSN in the cerebrospinal fluid to come into equilibrium with the serum after injection into the blood stream, while Bakay (1951) found that whereas it takes 12 - 24 hours for P32 injected into the blood stream to reach its maximal concentration in the brain, after injection in the Cisterna Magna the maximal concentration is reached in 30 - 60 minutes.

It must be mentioned at this point that certain fairly well defined portions of the brain are easily penetrated by substances in the blood. They are the hypophysis and certain related structures, and this property is no doubt associated with the endocrine functions that distinguish them from the central nervous system as a whole, (Wislocki and King, 1936; Bakay, 1952; Leduc and Wislocki, 1952).

It can be concluded from such facts that the principal part of the tissues of the brain and the spinal cord is bathed in an extracellular nutrient fluid independent of the blood system from which it is separated by an impermeable blood-brain barrier. Whether this extracellular fluid forms a continuous system with the cerebrospinal fluid as stated by Stern and Gautier (1922) is still a matter of discussion. Several authors have suggested that there is also a brain-cerebrospinal fluid barrier

(e.g. King, 1939; Friedemann, 1942; Aird and Strait, 1944) either lining the parenchyma of the brain around the perivascular spaces or resulting from the special character of the lipoid rich tissues. The functional importance of such a barrier seems very much exaggerated in the light of the work described by Katzenelbogen (1935), Wallace and Brodie (1939) and Bakay (1951).

The blood-brain barrier (in which term is included henceforth the "blood cerebrospinal fluid" barrier) is not absolutely impermeable; there has been a tendency to assume, mainly on the basis of experiments performed with acid and alkaline dyes, that positively charged particles can pass the barrier (Friedemann, 1942; Wislocki and Leduc, 1952) but this is not supported by the careful quantitative estimations of the distribution of various labelled cations cited earlier, (Hahn and Hevesy, 1941; Manery and Bale, 1941; Greenberg et al, 1943). However, it is known that lipoid soluble substances such as alcohol, acetone and chloroform penetrate into the cerebrospinal fluid rapidly (Mehrtens and Newman, 1933; Katzenelbogen, 1935) and that cocaine injected intravenously can be found in relatively high concentrations in the brain (Aird and Strait, 1944; Woods et al., 1951 b). The cerebrospinal fluid is in osmotic equilibrium with blood plasma (Fremont-Smith et al. 1931 a) and heavy water has

been found to penetrate easily all parts of the blood-brain barrier (Sweet et al. 1951; Bering 1952). These facts show that the internal environment of the central nervous system is separated from the blood and from other tissues by a diffusion barrier which is permeable to water and to many lipoid soluble substances. The principal components of the barrier are the choroid plexuses, the walls of the cerebrospinal capillaries and the arachnoid mater.

Since a parallel has been drawn between the sheaths of the central and the peripheral nervous systems, one cannot avoid the question whether the blood supply of the peripheral nerve has such properties as would justify the use of the term "blood nerve barrier". It would not seem unreasonable if another parallel could be drawn between the central nervous system and the peripheral nerve in this respect also. The possibility of a "blood nerve barrier" has apparently never been considered seriously, principally because the function of the peripheral nerve has usually been studied in specimens removed, for convenience, from their normal surroundings, and therefore devoid of their blood supply. If the blood supply was left intact, as has occasionally been done even in amphibian nerves (Parrack, 1940), this was to study the various features of nerve action under conditions as near to the normal as possible. It is, of course, evident that in contrast to the central nervous system, the peripheral nerve does not

have a large amount of extracellular fluid; the penetration of substances from the blood cannot be investigated by such a simple procedure as the lumbar or cisternal puncture. It is, no doubt, for such reasons that there is so little published evidence available.

In 1941, Manery and Bale examined the rate of entry of Na^{24} into several different tissues in the rabbit, and found that three of these were conspicuous by the long time necessary for final equilibrium. These tissues were the brain, the testes and the sciatic nerve (the only sample of peripheral nerve). Unfortunately, although these authors repeated the investigations of most tissues they only mentioned the sciatic nerve on one occasion. Euler et al.(1946) found that the penetration of 32P into muscle from the circulation was 2.5 times greater than into nerve in the cat. This is slender evidence, albeit the fruit of careful experiments and a sensitive technique. The experiments with dyes described earlier are suggestive but hardly conclusive. It can only be said therefore that much more work is required, with an exact quantitative technique like that of Manery and Bale, before a definite conclusion can be reached. Tracer experiments are in many ways ideal since very exact measurements can be made, and the units whose movements are studied are normal constituents of the tissues.

The present perfusion experiments might be considered as evidence against the presence of a "blood nerve barrier". That would be a mistake; when high concentrations of substances are used the effects of a relative barrier (there is no question of an absolute barrier) must be obscured, especially as the threshold of action in most cases is not known at all accurately.

The experiments of Dubner and Gerard (1939) and Rivkine (1950) show that the blood-brain barrier can be overlooked because its presence does not neutralize all blood changes. When the results obtained by Rivkine with the perfused frog brain are compared in detail with observations on the isolated brain (e.g. Libet and Gerard, 1939) then significant differences do become evident.

Similarly, although the nerve perfusion experiments gave rapid blocking times, a more exact analysis of the process of diffusion has shown that a "blood nerve" barrier probably exists. Whether this barrier is a selective agent comparable to the "blood brain" barrier or general impermeability of the capillary wall cannot at present be decided.

The description which has been given at some length of the "blood brain" barrier had for its main purpose to establish clearly its basic function, which is apparently to insulate the central nervous system from the circulating blood, as the ectodermal

sheaths insulate the whole nervous system from its surroundings. The concept of an insulated nervous system which thus arises will be seen to give a new meaning to the function of the perineurium.

It need hardly be pointed out that in a complex organism, it would seem essential that the nervous system should be in as stable as environment as possible if it is to serve its function with the utmost efficiency. A more constant environment is the equivalent of the stabilized power supply always provided in electrical circuits for sensitive triggers which control the whole network. Hence it is not surprising to find that an analogous concept has been presented before, although on rather different grounds. Trotter, in 1926, claimed that an insulated nervous system was so important to the animal that it would be an essential pre-requisite for higher development.

It is clear that such a "stabilized" internal environment would be reasonable and very profitable, but is there, in fact, any evidence that active stabilization is ever of any importance?

By far the most striking example of such stabilization has been described in insects recently by Hoyle (1952, 1953). Insect blood shows wide variations of ionic composition, with the intake of different foods. K, in particular, may be found in concentrations in which no vertebrate muscle or nerve could function. Hoyle has now demonstrated

that in the locust the entire nervous system is covered by an impermeable sheath which prevents wide changes in external K from interfering with nerve activity. Since the internal environment of the mammal as a whole is kept very much more constant than is the case with the insect, one might expect all evidence of any similar stabilization to have disappeared. That is not the case. Even in man, it seems that there may be a significant tendency for the environment of the central nervous system to remain constant. As the "blood brain" barrier is freely permeable to water, large changes in serum salts are mirrored in the cerebrospinal fluid but smaller changes tend to be neutralized. The fullest investigations to date of the respective values of Na in normal serum and cerebrospinal fluid (C.S.F.) are those given by Holley et al. (1951) and Shaw and Holley (1951). From their mean values a Na $\frac{\text{Serum}}{\text{C.S.F.}}$ ratio of 1.015 is obtained. The only large scale study of this ratio is that of Fremont-Smith et al. (1931a,b) and Dailey, (1931) who calculated its value in some 97 patients. Unfortunately, they did not give an adequate series of really normal controls for comparison. The significance of their average values is made questionable by the wide range of cases included, many of whom might well have disturbances of the blood brain barrier itself. (e.g. in various inflammatory or vascular disorders). Some of their figures, however, are suggestive:

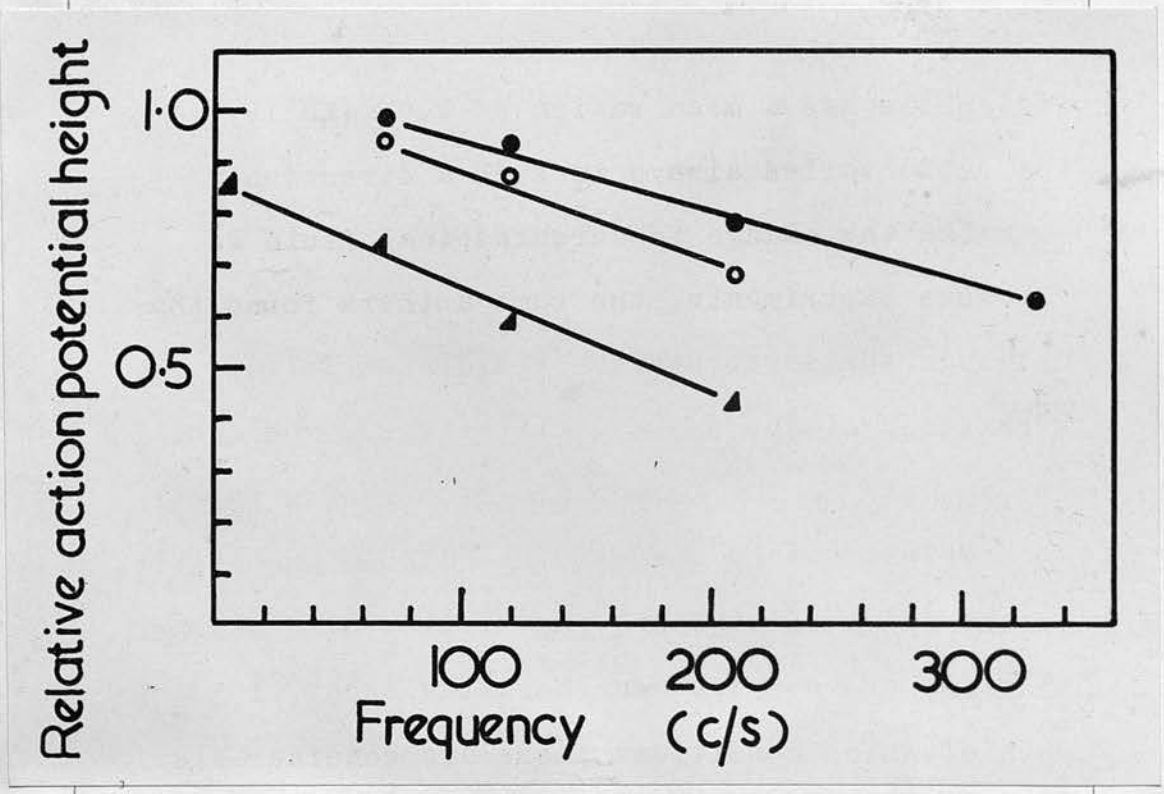

Fig.42. The relative height of the maximal action potential of a desheathed frog nerve in Na poor solution after 10 seconds of stimulation at several frequencies. The height of the action potential after 10 seconds at the same frequency in normal Ringer was always taken as unity.

The concentrations of the Na poor solutions were: 90% normal Na (●—●), 75% normal Na (o—o) and 50% normal Na (◢—◢). They were prepared by mixing suitable proportions of Ringer and isotonic choline chloride. The three straight lines were drawn arbitrarily.

12 cases with low serum Cl values had a mean Na $\frac{\text{Serum}}{\text{C.S.F.}}$ ratio of just under 0.95, whereas 28 cases with meningitis has a mean ration of 0.98 (Dailey, 1931). The ratio varied always in such a direction as to minimise the change in cerebrospinal fluid Na. In acute experiments, the same authors found that although the cerebrospinal fluid does follow variations of the serum qualitatively, the quantitative changes in the former are of a lesser magnitude.

Unfortunately there is no data in the literature on the Na $\frac{\text{Serum}}{\text{C.S.F.}}$ and K $\frac{\text{Serum}}{\text{C.S.F.}}$ ratios in Addison's disease, or in myxoedema (Margitay-Becht, 1937) in both of which conditions there are considerable alterations in serum electrolytes. In the results of experimental human salt deficiency given by McCance (1937, 1938) the two values of the ratio Na $\frac{\text{Serum}}{\text{C.S.F.}}$ were rather close to those of Dailey (1931) being 0.95 and 0.97.

Although a change in the $\frac{\text{Serum}}{\text{C.S.F.}}$ ratio of Na from 1.015 to 0.95 may seem trifling at first sight, it implies that the serum Na can decrease by nearly 7% with no change in the cerebrospinal fluid Na. A change of 7% may again seem insignificant; but in the course of some investigations into the effects of Na deficient solutions on frog nerves, it has been found that the ability of the nerve to conduct impulses at a relatively high frequency is extremely sensitive to even a small decrease (10%) in the Na concentration (fig.42). The early change in the

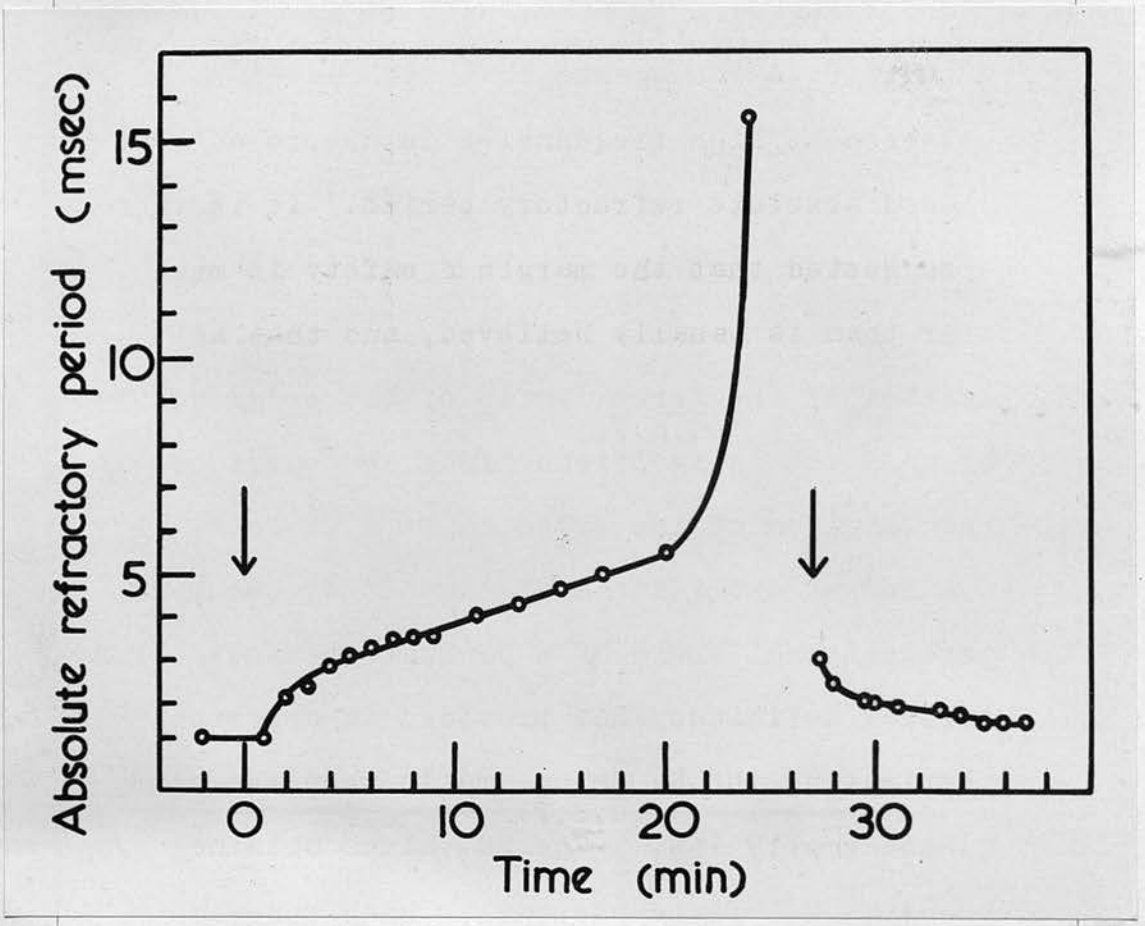

Fig. 43. The change in the absolute refactory period of a desheathed frog nerve placed in a Na free (sucrose) solution at the first arrow.
At the second arrow, the nerve was replaced into normal Ringer. The curve drawn is arbitrary.

absolute refractory period of a nerve in a Na-free solution (fig.43) suggests that at least part of the effect seen at high frequencies is due to an increased absolute refractory period. It is therefore suggested that the margin of safety is much smaller than is usually believed, and that an alteration of the $\frac{\text{Serum}}{\text{C.S.F.}}$ ratio of the order of 7% is by no means insignificant from the point of view of the function of the organism as a whole.

A recent examination of samples of serum and of cerebrospinal fluid of a patient with suspected suprarenal deficiency has provided an opportunity for estimating the Na $\frac{\text{Serum}}{\text{C.S.F.}}$ ratio when the serum Na is abnormally low. The Na values obtained by means of an Eel flame photometer were substantially higher than those found earlier by a chemical method in the clinical laboratory, but the $\frac{\text{Serum}}{\text{C.S.F.}}$ ratio was exactly the same, i.e. 0.885. This is equivalent to a 13% fall in the serum Na with no change in the C.S.F. Na. In fact, the C.S.F. Na value given by the flame photometer was practically normal. The tendency of the flame photometer Na values to be somewhat higher than those obtained by chemical estimations have been described by Shaw and Holley (1951).

Another manifestation of the blood brain barrier was described by McCance and Watchorn (1937), who found that the changes in serum Ca which accom-

panied overbreathing tetany, were not reproduced in the cerebrospinal fluid.

The stabilization of the internal environment of the nervous system therefore seems a very real thing which can be detected even in man (in the cerebrospinal fluid). This regulation may be of considerable importance in relation to many pathological conditions..

One of the most intriguing, and perhaps significant, associations is that between epilepsy and convulsive states in general, and the permeability of the blood brain barrier. Aird and Strait (1951) showed that one action of the drug dilatin used in the treatment of epilepsy, is to lower the permeability of the blood brain barrier. The lowering of the convulsive threshold is in some way connected with an increased permeability of the "blood brain" barrier, as in post concussional states (Aird et al., 1952); it can be reversed by certain dyes (e.g. Trypan red) which decrease its permeability (Aird and Strait, 1944).

The increases in serum and C.S.F. Na (Holley et al., 1953) associated with hypertension are interesting phenomena which may be influenced by the properties of the blood brain barrier.

The well known problem of the transport of neurotropic viruses and of tetanus toxin remains to be solved, but the importance of the insulation of the nervous system with regard to tetanus toxin is

well established, (Friedemann, 1942).

The exact relationship between nervous tissue and its internal environment can only be guessed at for the time being. It cannot be doubted that from the normal relationship, or from its disturbance, arise many puzzling phenomena, such as the various effects of anoxia, and repeated washing, on the peripheral nerve (Aykut, 1952), or the swelling of desheathed nerves (Lorente de No, 1952d), or brain slices (Wessberge, 1914a,b; Elliot, 1946; Stern et al. 1949), in many solutions.

In conclusion, no apology is made for the rather extensive reviews of the evidence for the blood-brain barrier, the ectodermal origin of the sheaths, and intraneural fluid pathways, because these are controversial subjects, and not universally accepted facts which could be taken for granted. It is hoped that from the matter which may have seemed irrelevant at times, a clear conception of the nature, the properties and the significance of the peripheral nerve sheath will have emerged. It is probably an essential part of an impermeable ectodermal covering of the entire nervous system, which, by supplementing processes of selective absorption or secretion, may serve to stabilize its internal environment.

8. A large number of counts of perfused blood vessels in the frog sciatic nerve give a mean concentration of vessels of the order of 50/mm^2.

9. A variety of histological examinations of frog sciatic nerves suggest, among other things, that it is the perineurium which is removed by desheathing.

10. The diffusion constants of Na and K in perfused and non-perfused nerves, and also in desheathed nerves (Na), were estimated. The relative values of the diffusion constants were:

$$\frac{\text{Perfused}}{\text{Non-perfused}} \quad \begin{matrix} \text{Na} & \text{K} \\ = 13 & 4 \end{matrix} \qquad \frac{\text{Desheathed}}{\text{Perfused}} \quad \begin{matrix} \text{Na} \\ = 4 \end{matrix}$$

11. It is concluded that the frog nerve perineurium is a barrier to the diffusion of electrolytes.

12. After a review of the physiology and development of the leptomeninx, of the blood-brain barrier, and of intraneural pathways, it is suggested that the perineurium is only a part of an extensive system of ectodermal sheaths which cover the entire nervous system.

13. Evidence is given and reviewed that these sheaths, together with the blood-brain barrier and a hypothetical blood-nerve barrier, may serve to stabilize the internal environment of the nervous system.

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REFERENCES

ABEL,J.J., FIROR,W.M., & CHALIAN,W. (1938)."Researches on tetanus: 9. Further evidence to show that tetanus toxin is not carried to central neurons by way of the axis cylinders of motor nerves." Johns.Hopk.Hosp.Bull 63, 373-403.

ADAMS,W.E. (1942). "The blood supply of nerves." J.Anat.,Lond. 76, 323-341.

AIRD,R.B. & STRAIT,L. (1944) "Protective barrier of the central nervous system; an experimental study with Trypan Red." Arch.Neurol.Psychiat., Chicago. 51,54-66.

AIRD,R.B., STRAIT,L.S., ZEALAR,D. & HRENOFF,M. (1952) "Neurophysiological studies on cerebral concussion" J.Neurosurg, 9, 331-347.

AIRD,R.B. & STRAIT,L. (1951). "The mode of action of diphenylhydantoinate (dilantin) in epilepsy." J.Pharmacol. 103, 136-146.

AYKUT,R. & WINTERSTEIN,H. (1950 a). "Das Problem der anoxyschen Erholung asphyktischer Organe besonders des Nerves". Arch. int. Pharmacodyn. 81, 99-110.

AYKUT,R. & WINTERSTEIN,H. (1950 b). "Anoxybiose des Froschnerven in Sauerstoffreien Lösungen". Arch. int.Pharmacodyn. 81, 222-229.

BAKAY,L. (1951). "Studies on the blood-brain barrier with radioactive phosphorus." Arch.Neurol.Psychiat. Chicago. 66, 419-426.

BAKAY,L. (1952). "Studies on blood-brain barrier with radioactive phosphorus: 2. Hypophysis and hypothalamus in man." Arch.Neurol.Psychiat. Chicago 68, 629-640.

BERING,E.A. (1952) "Water exchange of central nervous system and cerebrospinal fluid." J.Neurosurg. 9, 275-287.

BERNSTEIN,J. (1912) "Elektrobiologie" Braunschweig:Vieweg.

BISHOP,G.H. (1928a). "The relation between the threshold of nerve response and polarization by galvanic current stimuli". Amer.J.Physiol. 84, 417-436.

BISHOP,G.H. (1928b). "The effect of nerve reactance on the threshold of nerve during galvanic current flow". Amer.J.Physiol. 85, 417-431.

BISHOP,G.H. (1932). "Action of nerve depressants on potential". J. cell. comp. Physiol. 1, 177-194.

BISHOP,G.H., ERLANGER,J. & GASSER,H.S. (1926). "Distortion of action potential as recorded from the nerve surface". Amer.J.Physiol. 78, 592-609.

BRIERLEY,J.B. (1950). "The penetration of particulate matter from the cerebrospinal fluid into the spinal ganglia, peripheral nerves and perivascular spaces of the central nervous system." J.Neurol. 13, 203-215.

BRIERLEY,J.B. & FIELD,E.J. (1948). "The connexions of the spinal subarachnoid space with the lymphatic system." J.Anat.,Lond. 82, 153-166.

BRIERLEY,J.B. & FIELD,E.J. (1949). "The fate of an intraneural injection as demonstrated by the use of radio-active phosphorus." J.Neurol. 12, 86-99.

BÜLBRING,E. & WHITTERIDGE,D. (1941). "The effect of adrenaline on nerve action potentials."J.Physiol. 99, 201-207.

BUNZEN,-. (1807). "Einige neue glavanishe Versuche und einige Athmungsversuche". Gilbert's Annalen der Physik. 25, 147-163.

BURTON-OPITZ,R. (1902). "A comparative study of the viscosity of the blood." Amer.J.Physiol. 7,243-260.

BURTON-OPITZ,R. (1920). "A textbook of Physiology". Philadelphia and London: Saunders.

CAUSEY,G. & PALMER,E. (1953). "The epineural sheath of a nerve as a barrier to the diffusion of phosphate ions." J.Anat., Lond. 87, 30-36.

CHAMBERS,R. & ZWEIFACH,B.W. (1940) "Capillary endothelial cement in relation to permeability." J.cell.comp.Physiol. 15, 255-272.

CHAMBERS,R. & ZWEIFACH,B.W. (1944) "Topography and function of the mesenteric capillary circulation." Amer.J.Anat. 75, 173-205.

CHAMBERS,R. & ZWEIFACH,B.W. (1947) "Intercellular cement and capillary permeability." Physiol.Rev. 27, 436-463.

CHURCHILL,E.D., NAKAZAWA,F. & DRINKER,C.K. (1927). "The circulation of body fluids in the frog". J.Physiol. 63, 304-308.

COHEN,R.A. & GERARD,R.W. (1933). "The anoxic recovery of asphyxiated nerve". J.cell.comp.Physiol. 3, 425-436.

COLE,K.S. & CURTIS,H.J. (1936). "Electric impedance of nerve and muscle". Cold Spr.Harb.Symp.quant. Biol. 4, 73-87.

COWIE,D.B., FLEXNER,L.B. & WILDE,W.S. (1949) "Capillary permeability: rate of transcapillary exchange of chloride in the guinea pig as determined with radio chloride." Amer.J.Physiol. 158, 231-236.

CRESCITELLI,F. (1951). "Nerve sheath as a barrier to the action of certain substances". Amer.J.Physiol. 166, 229-240.

CRESCITELLI,F. (1952a) "Interaction between certain nerve blocking-drugs and sodium ions". Fed.Proc. 11, 29.

CRESCITELLI,F. (1952b) "Some features in responses of different nerve fibre types to a deficiency of sodium." Amer,J.Physiol. 169, 1-10.

CRESCITELLI,F. (1952c). "Modification in responses to sodium of nerve fibres treated with drugs". Amer.J.Physiol. 169, 638-648.

CRESCITELLI,F. & GEISSMAN,T.A. (1951). "Certain effects of antihistamine and related compounds on frog nerve fibres". Amer.J.Physiol. 164, 509-519.

CUSHING,H. (1901). "Concerning the poisonous effect of pure sodium chloride solutions upon the nerve-muscle preparation." Amer,J.Physiol. 6, 77-90.

DAILEY,M.E. (1931) See final page.

DANIELLI,J.F. (1940). "Capillary permeability and oedema in the perfused frog." J.Physiol.98, 109-129.

DANIELLI,J.F. (1941) "A method for estimating the fraction of the volume of a muscle contained in the vascular system." J.Physiol. 100, 239-245.

DANIELLI,J.F. & DAVSON,H. (1941). "The volume of the vascular system and penetration of sugars from the vascular system into the intercellular space." J.Physiol. 100, 246-255.

DRINKER,C.K. (1927). "The permeability and diameter of the capillaries in the web of the brown frog (R.Temporaria) when perfused with solutions containing pituitary extract and horse serum." J.Physiol. 63, 249-269.

DUBNER,H.H. & GERARD,R.W. (1939) "Factors controlling brain potentials in the cat". J.Neurophysiol. 2, 142-152.

DU BOIS-REYMOND,E. (1848-84). "Untersuchungen über thierische Elektricität". 2, 275-282. Berlin: Reimer.

EBBECKE,U. (1926). "Uber die Polarization im Nerven und Muskel und ihre Messung". Pflüg.Arch. ges. Physiol. 212, 121-135.

ECKARD,C. (1851). "Die chemische Reizung der motorischen Froschnerven". Z.rationelle Medicin, N.F. 1, 303-328.

ECKER,A & WIEDERSHEIM,R. (1899). "Anatomie des Frosches". 2, p.123, ed. GAUPP,E. Braunsweig: Vieweg.

EICHLER,O., LINDER,F. & SCHMEISER,K. (1951). "Über die Bildung von Liquor im Lumbalraum, nachgewiesen mit Radionatrium." Klin.Wschr. 29, 9-12.

ELLIOT,K.A.C. (1946) "Swelling of brain slices and the permeability of brain cells to glucose." Proc.Soc.exp.Biol., N.Y. 63, 234-236.

ELMAN,J. (1923). "Spinal arachnoid granulations with especial reference to the cerebrospinal fluid." Johns Hopk.Hosp.Bull. 34, 99-104.

ERLANGER,J. andBLAIR,E.A. (1934) see final page.

ERLANGER,J. & BLAIR,E.A. (1938). "The action of isotonic, salt free solutions on conduction in medullated nerve fibres". Amer.J.Physiol. 124, 341-359.

ERLANGER,J. BISHOP,G.H. & GASSER,H.S. (1926). "The action potential waves transmitted between the sciatic nerve and its spinal roots". Amer.J. Physiol. 78, 574-591.

EULER,H., EULER,U.S. & HEVESY,G. (1946) "The effect of excitation on nerve permeability." Acta physiol. scand. 12, 261-267.

FENG,T.P. & GERARD,R.W. (1930). "Mechanism of nerve asphyxiation: with a note on the nerve sheath as a diffusion barrier". Proc.Soc.exp.Biol. N.Y. 27, 1073-1076.

FENG,T.P., HSU,C.H. & LIU,Y.M. (1950a) "The mechanism of the recovery of nerve asphyxiated in nitrogen when washed with O_2 free Ringer". Chin.J.Physiol. 17, 247-258.

FENG,T.P. HSU,C.H. & LIU,Y.M. (1950b) "Correlation of potassium movement into and out of the nerve with its depolarization and repolarization". Chin. J. Physiol. 17, 281-286.

FENG,T.P. & LIU,Y.M. (1949a) "The connective tissue sheath of the nerve as effective diffusion barrier". J.cell.comp.Physiol. 34, 1-16.

FENG,T.P. & LIU,Y.M. (1949b). "The concentration effect relationship in the depolarization of amphibian nerve by potassium and other agents". J.cell.comp.Physiol. 34, 33-42.

FENN,W.O. (1928) "The carbon dioxide dissociation curve of nerve and muscle." Amer.J.Physiol. 85, 207-223.

FENN,W.O., COBB,D.M. & HEGNAUER,A.H. & MARSH,B.S. (1934). "Electrolytes in nerve". Amer.J.Physiol. 110, 74-96.

FLEXNER,L.B. (1929). "The development of the meninges in amphibians: A study of normal and experimental animals." Contr.Embryol.Carneg.Instn.No.110,publ. 394, 20, 31-50.

FLEXNER,L.B. (1934). "The chemistry and nature of the cerebrospinal fluid." Physiol. Rev. 14, 161-187.

FLEXNER,L.B., COWIE,D.B. & VORSBURGH,G.J. (1948). "Studies on capillary permeability with tracer substances." Cold Spr.Harb.Symp.quant.Biol. 13, 88-97.

FREMONT-SMITH, et. al. 1931 (a,b) - see final page.

FRIEDEMANN,U. (1942). "Blood brain barrier." Physiol. Rev. 22, 125-145.

FRIEDEMANN,U., HOLLANDER,A., & TARLOV,I.M. (1941). "Investigations on the pathogenesis of Tetanus. 3." J. Immunol. 40, 325-364.

GERARD,R.W. (1927) "Studies on nerve metabolism: 2; Respiration in Oxygen and Nitrogen." Amer.J. Physiol. 82, 381-404.

GERARD,R.W. (1930). "The response of nerve to oxygen lack." Amer.J.Physiol. 92, 498-541.

GERARD,R.W. (1932). "Nerve metabolism". Physiol.Rev. 12, 469-592.

GILDEMEISTER,M. (1928). "Die passiv- elektrischen Erscheinungen im Tier- und Pflanzenreich". In "Handbuch der normalen und pathologischen Physiologie". Band 8, Teil 2, 679-702. Berlin: Springer.

GREENBERG,D.M., AIRD,R.B., BOELTER,M.D.D., CAMPBELL, W.W., COHN,W.E. & MURAYAMA,M.M. (1943). "A study with radioactive isotopes of the permeability of the blood-cerebrospinal fluid barrier to ions." Amer.J.Physiol. 140, 47-64.

GROVES,E.W. (1893). "On the chemical stimulation of nerves". J.Physiol. 14, 221-232.

GRÜTZNER,P. (1878). "Über verschiedene Arten der Nervenerregung. 1. Über die Einwirkung von Wärme und Kälte auf Nerven". Pflüg.Arch.ges.Physiol. 17, 215-238.

HAHN,L. & HEVESY,G. (1941). "Rate of penetration of ions through the capillary wall." Acta physiol. scand. 1, 347-361.

HARLESS,E. (1846). "Über die funktionell verschiedenen Partien des Rückenmarcks der Amphibien". Müller's Arch.Anat.Physiol.viss.Med. 74-95.

HARRISON,R.G. (1924). "Neuroblast versus sheath cell in the development of peripheral nerves." J.comp.Neurol. 37, 123-196.

HARVEY,S.C. & BURR,S.H. (1926). "The development of the meninges." Arch.Neurol.Psychiat.Chicago. 15, 545-567.

HARVEY,S.C., BURR,S.H. & VAN CAMPENHOUT,E. (1933). "Development of the meninges." Arch.Neurol. Psychiat., Chicago. 29, 683-690.

HASSIN,G.B. (1947). "The cerebrospinal fluid pathways" J.Neuropath. 6, 172-176.

HERMANN,L. (1867). "Grundriss der Physiologie des Menschen". p. 285, Berlin: Hirschwald.

HERMANN,L. (1879). "Handbuch der Physiologie des Nervensystems". Band 1, p. 96 - 97, Leipzig:Vogel.

HIATT,E.P. (1939). "Replacement of chlorides in tissues and body fluids of dogs by nitrates." Amer.J.Physiol. 126, P.533.

HILL,A.V. (1928). "The diffusion of oxygen and lactic acid through tissues." Proc.Roy.Soc. B.104, 39-96.

HILL,A.V. (1948) "On the time required for diffusion and its relation to processes in muscle." Proc.Roy. Soc. 135, 446-453.

HODGKIN,A.L. (1951). "The ionic basis of electrical activity in nerve and muscle".Biol.Rev. 26,339-409.

HOLLEY,H.L.,ELLIOT,H.C. & HOLLAND,C.M. (1951). "Serum sodium values in essential hypertension." Proc.Soc.exp.Biol.,N.Y. 77, 561-563.

HORSTER,H. & WHITMAN,L. (1931). "Die Methode der intraneuralen Injektion." Z.Hyg.InfektKr. 113, 113-124.

HOWARTH,F. & COOPER,E.R.A. (1949). "Departure of substances from the spinal theca." Lancet, 2, 937-940.

HOYLE,G. (1952). "High blood potassium in insects in relation to nerve conduction," Nature, Lond. 169, 281-282.

HOYLE,G. (1953) "Potassium ions and insect nerve muscle." J.exp.Biol. 30, 121-135.

HUTTER,O.F. (1949). "Effect of ~~demarcation~~ denervation on development of local tetanus". J.Physiol.112, 1 P.

HUXLEY,A.F. & STÄMPFLI,R. (1949). "Evidence for saltatory conduction in peripheral myelinated nerve fibres". J.Physiol. 108, 315-339.

HUXLEY,A.F. & STAMPFLI,R. (1951). "Effect of potassium and sodium on resting and action potentials of single, myelinated nerve fibres". J.Physiol. 112, 496-508.

HYMAN,C & CHAMBERS,R. (1943). "Effect of adrenal cortical compounds on oedema formation of frogs' hind limbs." Endocrinology 32, 310-318.

IWANOW,G. (1928). "Über die Abflusswege aus den submeningealen Räumen des Rückenmarks." Z.ges.exp.Med. 58, 1-21.

IWANOW,G. (1929). "Über die Abflusswege aus den Subarachnoidalräumen des Gehirns und Rückenmarks und über die Methodik ihrer intravitalen Untersuchung." Z.ges.exp.Med. 64, 356-375.

IWANOW,G. & ROMODANOWSKY,K. (1928). "Über den anatomischen Zusammenhang der cerebralen und spinalen submeningealen Räume mit dem Lymphsystem."

JACOBS,M.H. (1935). "Diffusion processes." Ergebn.Biol. 12, 1-160.

JOHNSON,LL., SHAW,C.W., GALEN,W.P. & HOLLEY,H.L.(1953) "Cerebrospinal fluid values in essential hypertension." J.Lab.clin.Med. 41, 287-289.

KATO,G. (1936). "On the excitation, conduction and narcotization of single nerve fibres". Cold.Spr. Harb. Symp. quant. Biol. 4, 202-213.

KATZENELBOGEN,S. (1935). "The cerebrospinal fluid and its relation to the blood." Baltimore: Johns Hopkins Press,

KEY,A & RETZIUS,G. (1873). "Studien in der Anatomie des Nervensystems". Arch.mikr.Anat. 9, 308 - 386.

KEY,A. & RETZIUS,G. (1876). "Studien in der Anatomie der Nervensystems und des Bindegewebes". Band 2, Stockholm.

KING,L.S. (1939) "The hemato-encephalic barrier." Arch.Neurol.Psychiat., Chicago. 41, 51- 72.

KROGH,A. (1919a) "The rate of diffusion of gases through animal tissues, with some remarks on the co-efficient of invasion." J.Physiol.52, 391-408.

KROGH,A. (1919b). "The number and distribution of capillaries in muscles with calculation of the oxygen pressure head necessary for supplying the tissue." J. Physiol. 52, 409-415.

KROGH,A. (1919c) "The supply of oxygen to the tissues and the regulation of the capillary circulation." J. Physiol. 52, 457-474.

KROGH,A. (1922). "The anatomy and physiology of capillaries." Silliman Memorial Lectures, Yale Univ. Press, New Haven.

KÜHNE,M.W. (1859). "Sur l'irritation chimique des nerfs et des muscles". C.R.Acad.Sci., Paris. 48, 406-409.

LAIDLAW,G.F. (1929). "Silver staining of the skin and of its tumours." Amer.J.Path. 5, 239-248.

LAIDLAW,G.F. (1930). "Silver staining of the endo-neural fibres of the cerebrospinal nerves".Amer. J. Path. 6, 435- 443.

LANDIS,E.M. (1927) "Microinjection studies of capillary permeability." Amer.J.Physiol. 82, 217-238.

LÄWEN,A.H. (1904). "Quantitative Untersuchungen über die Gefässwirkung von Suprarenin." Arch.exp.Path. Pharmak. 51, 415-441.

LEAR,T. & EDWARDS,E.A. (1933) "The subdural space and its linings". Arch.Neurol.Psychiat.,Chicago. 29, 691-701.

LEDUC,E.H. & WISLOCKI,G.B. (1952). "The histochemical localization of acid and alkaline phosphatases, non specific esterases and succinic dehydrogenases in the structures comprising the hemato-encephalic barrier of the rat." J.comp.Neurol. 97, 241-279.

LEVINSON,A. (1919). "Cerebrospinal fluid in health and disease." London: Henry Kimpton.

LIBET,B. & GERARD,R.W. (1939). "Control of the potential rhythm of the isolated frog brain." J.Neurophysiol. 2, 153-169.

LONGET,F.A. (1842). "Anatomie et physiologie du système nerveux de l'homme et des animaux vertébrés." 1, 136. Paris.

LORENTE DE NO,R. (1947). "A study of nerve physiology" Studies from the Rockefeller Institute. vol.131 & 132.

LORENTE DE NO, R. (1948). "Quarternary ammonium ions and sodium ions in nerve physiology". Johns Hopk. Hosp. Bull. 83, 497-529.

LORENTE DE NO,R. (1949). "On the effect of certain quarternary ammonium ions upon frog nerve". J. cell,comp.Physiol. 33, (suppl.).

LORENTE DE NO,R. (1950). "The ineffectiveness of the connective tissue sheath as a diffusion barrier". J.cell.comp.Physiol. 35, 195-240.

LORENTE DE NO,R. (1951a) "Equilibrium of frog nerve with different external concentrations of sodium ions". J.gen.Physiol. 35, 145-182.

LORENTE DE NO,R. (1951b) "On the existence of a gradient of sensitivity to the lack of sodium in the spinal roots of the bull frog". J.gen.Physiol. 35, 183-201.

LORENTE DE NO,R. (1952a) Public lecture, University College, London.

LORENTE DE NO,R. (1952b) Personal communication.

LORENTE DE NO,R. (1952c) Personal communication.

LORENTE DE NO,R. (1952d) "Observations on the properties of the epineurium of the frog nerve." Cold Spr.Harb.Symp.quant.Biol. 17, 299-314.

LULLIES,H. (1930) "Über die Polarisation in Geweben. 2. Die Polarisation in Nerven 1". Pflüg.Arch.ges. Physiol. 225, 69-86.

LUNDBERG,A. (1951a). "On the effect of temperature on the depolarization of frog nerve fibres". Acta. physiol.scand. 22, 348-364.

LUNDBERG,A. (1951c). "Electrotonus in frog spinal roots and sciatic trunk". Acta physiol.scand. 23, 234-262.

McCANCE,R.A. (1937). "The changes in the plasma and cells during experimental human salt deficiency." Biochem.J. 31, 1278-1284.

McCANCE,R.A. (1938) "The effect of salt deficiency in man on the volume of the extracellular fluids and on the composition of sweat, saliva, gastric juice and cerebrospinal fluid." J.Physiol. 92, 208-218.

McCANCE,R.A. & WATCHORN,E. (1937) "Overbreathing tetany: changes in the calcium of serum, serum ultrafiltrates, and cerebrospinal fluid." Lancet p.200.

MACDONALD,J.S. (1900). "The demarcation current of mammalian nerve". Proc.Roy.Soc. 67, 310-328.

MACDONALD, J.S. (1902). "The injury current of nerve" Thompson Yates Laboratories Report. 4, part 2, 213-347.

MANERY,J.F. & BALE,W.F. (1941). "The penetration of radioactive sodium and phosphorus into the extra- and intracellular phases of tissue." Amer.J.Physiol. 132, 215-231.

MARGITAY-BECHT,A. (1937) "Der Natriumgehalt des Blutserums bei Myxödem." Klin.Wschr. 16, 1353-1355

MASSON,P. (1942). "Tumeurs encapsulees et begnines des nerfs. "Rev.canad.Biol. 1, 209-343.

MEHRTENS,H.G. & NEWMAN,H.W. (1933). "Alcohol injected intravenously." Arch.Neurol.Psychiat. Chicago. 30, 1092-1099.

MEYER,H. & RANSOM,F. (1903). "Untersuchungen über den Tetanus." Arch.exp.Path.Pharmak. 49, 369-416.

MIES,H. (1926). "Über die Gefäss versorgung des Nervus Ischiadicus beim Frosche, ihre physiologische und pharmakologische Bedeutung." Pflüg. Arch.ges.Physiol. 214, 532-536.

MOND,R. & AMSON,K. (1928). "Über die Ionenpermeabilität des quergestreiften Muskels." Pflüg.Arch.ges. Physiol. 220, 69-81.

MULDER,J.D. (1938). "An experimental and critical study in the spreading of foreign substances in the nerve." Acta neerl.Morph. 1, 288-301.

MULLINS,L.J. (1950) "Uptake of phosphate by frog axons". Fed.Proc. 9, 93.

MUSKAT,M. (1937) "The flow of homogeneous fluids through porous media." New York and London: McGraw-Hill.

NETTER,H. (1926). "Über die Permeabilitatseigenschaften der Nervenhüllen". Pflüg.Arch.ges.Physiol. 215, 373-385.

OVERTON,E. (1902a) "Beiträge zur allgemeinen Muskel- und Nervenphysiologie. 1. Über die osmotischen Eigenschaften der Muskeln." Pflüg.Arch.ges.Physiol. 92, 115-280.

OVERTON,E. (1902b). "Beiträge zur allgemeinen Muskel- und Nervenphysiologie. 2. Über die Entbehrlichkeit von Natrium- (oder Lithium-) Ionen für den Contraktionsact des Muskels." Pflüg.Arch.ges. Physiol. 92, 346-386.

OVERTON,E. (1904). "Beiträge zur allgemeinen Muskel- und Nervenphysiologie. 3. Studien über die Wirkung der Alkali- und Erdalkalisalze auf Skelettmuskeln und Nerven." Pflüg.Arch.ges. Physiol. 105, 176-290.

PAPPENHEIMER,J.R., RENKIN,E.M. & BORRERO,L.M. (1952) "Filtration, diffusion and molecular sieving through peripheral capillary membranes, a contribution to the pore theory of capillary permeability". Amer.J.Physiol. 167, 13-46.

PARRACK,H.O. (1940). "Excitability of the excised and circulated frog's sciatic nerve." Amer.J.Physiol. 130, 481-495.

PLENCK,H. (1927). "Über argyrophile Fasern (Gitterfasern) und ihre Bildungszellen." Ergebn.Anat. EntwGesch. 27, 302-412.

PONOMAREFF,A.W. (1927). "Sur les conditions qui modifient la vitesse de propagation de la toxine tetanique dans le nerf." C.R.Soc.Biol.,Paris. 97, 503-504.

RANVIER,M.L. (1878). "Leçons sur l'histologie du systeme nerveux." Tome 1, 179-238. Paris: Savy.

de RENYI,G.S. (1932). "Architecture of the nerve cell as revealed by microdissection." In COWDRY,E.W., "Special Cytology". 2, 1369-1402. New York:Hoeber.

RICE,L.H. & DAVIS,H. (1928). "Uniformity of narcosis in peripheral nerve." Amer.J.Physiol. 87, 73-84.

von RIJSSEL,T.G. (1946). "Circulation of cerebrospinal fluid in Carassius Gibelio." Arch.Neurol. Psychiat.Chicago. 56, 522-543.

RIVKINE,A. (1950) "Action d'une variation de potassium ou de calcium sur l'electrocérébrogramme de la grenouille." Arch.int.Physiol. 57, 245-266.

RÖSSEL,W. (1943). "Der Einfluss der Nervenhüllen auf die elektrolytische Polarisation und die Erregbarkeit de Froschischiadicus." Pflüg.Arch. ges.Physiol. 246, 543-552.

ROUGHTON,F.J.W. (1952). "Diffusion and chemical reaction velocity in cylindrical and spherical systems of physiological interest." Proc.Roy.Soc. B. 140, 203-229.

SASLOW,G. (1938). "The relation between the oxygenation of fluids and the occurence of oedema in the perfused frog web." Amer.J.Physiol. 124, 360-368.

SCHMITT,O.H. & STEWART,P.A. (1950). "Evidence for physiological activity in nerve membrane in response to subthreshold stimulation". Fed.Proc. 9, p. 113.

SCHMITZ,W. & SCHAEFER,H. (1933a). "Über den Nervenaktionsstrom und die positive Nachschwankung." Pflüg.Arch.ges.Physiol. 232, 7-19.

SCHMITZ,W. & SCHAEFER,H. (1933b). "Zum Nachweis der Polarisationskapazität am Nerven." Pflüg.Arch.ges. Physiol. 232, 20-23.

SCHOEPFLE,G.M. & SUSMAN,N. (1950). "Physical significance of strength-duration curve for excitation of nerve." J.Neurophysiol. 13, 289-293.

SHAW,C.W. & HOLLEY,H.L. (1951). "Sodium and potassium concentration in human cerebrospinal fluid." J.Lab.clin.Med. 38, 574-576.

STÄMPFLI,R. (1952). "Bau und Funktion isolierter markhaltiger Nervenfasern." Ergebn.Physiol. 1952, 70-165.

STERN,J.R., EGGLETON,L.V., HEMS,R. & KREBS,H.A.(1949). "Accumulation of Glutamic acid in isolated brain tissue." Biochem.J. 44, 410-418.

STERN,L. & GAUTIER,R. (1922)."Recherches sur le liquide céphalo-rachidien. 2. Les rapports entre le liquide céphalo-rachidien et les éléments nerveux de l'axe cérébro-spinal". Arch.int. Physiol. 17, 391-448.

STERZI,G. (1902). "Recherches sur l'anatomie comparée et sur l'ontogenèse des méninges." Arch.ital.Biol. 37, 257-269.

SULLIVAN,W.E. & MORTENSEN,O.A. (1934). "Visualization of the movement of a brominized oil along peripheral nerves." Anat.Rec. 59, 493-499.

SUNDERLAND,S. (1945). "Blood supply of the nerves of the upper limb in man." Arch.Neurol.Psychiat. Chicago. 53, 91-115.

SWEET,W.H., SELVERSTONE,B., SOLOWAY,S. & STETTEN,D. (1951). "Studies of formation, flow and absorbtion of cerebrospinal fluid: II. Studies with heavy water in the normal man." In "Surgical forum, Amer.Coll.Surg." Philadelphia and London: Saunders. 376-381.

TARLOV,I.M. (1937). "1. Nature of the junction between the central and peripheral nervous systems. Structure of the nerve root." Arch. Neurol.Psychiat. Chicago. 37, 555-583.

TASAKI,I. (1939a) "The strength duration relation of the normal polarized and narcotized nerve fibres." Amer.J.Physiol. 125, 367-379.

TASAKI,I. (1942). "Das Schwellenabsinken bei Reizung einer Nervenfaser mit kurzen Stromstössen." Pflüg.Arch.ges.Physiol. 245, 665-679.

TEALE,F.H. & EMBLETON,D. (1919). "Studies in infection. 2. The paths of spread of bacterial exotoxins with special reference to tetanus toxin." J.Path.Bact. 23, 50-68.

TIGERSTEDT,R. (1921). "Die Physiologie des Kreislaufes." 2, p. 106. Berlin and Leipzig.

TRENDELENBURG.P. (1910). "Bestimmung der Adrenalingehaltes im normalen Blut sowie beim Abklingen der Wirkung einer intravenösen Adrenalininjektion mittels physiologischer Messmethode."Arch.exp.Path. Pharmak. 63, 16-176.

TROTTER,W. (1926). "The insulation of the nervous system." pp. 47-70 in "The collected papers of Wilfred Trotter, F.R.S." 1941. London: Humphrey Milford.

TSCHIRGI,R.D. & TAYLOR,J.L. (1953). "Influence of various agents on the cerebrospinal fluid potential." Fed.Proc. 12, 145.

USSING,H.H. (1949). "The distinction by means of tracers between active transport and diffusion." Acta physiol.scand. 19, 43-56.

VERWORN,M. (1900). "Ermüdung, Erschöpfung und Erholung der nervösen Centra des Ruckenmarkes." Arch.Anat.Physiol.,Lpz. 1900, Suppl. 152-176.

WALLACE,G.B. & BRODIE,B.B. (1939). "The distribution of iodide, thiocyanate, bromide and chloride in the central nervous system and spinal fluid." J.Pharmacol. 65, 220-226.

WALLACE,G.B. & BRODIE,B.B. (1940). "The passage of bromide, iodide and thiocyanate into and out of the cerebrospinal fluid." J.Pharmacol. 68, 50-55.

WEED,L.H. (1914). "Studies on cerebrospinal fluid: 2. The theories of drainage of cerebrospinal fluid with an analysis of the methods of investigation. 3. The pathways of escape from the subarachnoid spaces with particular reference to the arachnoid villi. 4. The dual source of cerebrospinal fluid." J.med.Res. 31, 21-117.

WEED,L.H. (1938). "Meninges and cerebrospinal fluid." J.Anat., Lond. 72, 181-215.

WESSBERGE,H. (1914b) "Nouvelles recherches sur les variations de poids subies par des encéphales d'oiseaux, immergés dans des solutions de NaCl, de $CaCl_2$ et de saccharose." C.R.Soc.Biol.,Paris. 77, 70-72.

WESSBERGE,H. (1914a) "Variations de poids subies par la substance blanche et la substance grise du cerveau du cheval immergées dans des solutions de NaCl, Kcl et $CaCl_2$. C.R.Soc.Biol.,Paris.77,194-196.

WHITE,H.L. (1924). "On glomerular filtration." Amer. J.Physiol. 68, 523-529.

WINKELMANN,N.W. (1949)."Peripheral nerve and root disturbances following vaccination against smallpox." Arch.Neurol.Psychiat. Chicago. 62, 421-438.

WISCHNEWSKY,A.S. (1928). "Über die Bedingungen einer verschiedenen Schnelligkeit der Fortbewegung von Farbstoffen im Nerven." Z,ges.exp.Med.61, 107-113.

WISLOCKI,G.B. & KING,L.S. (1936). "The permeability of the hypophysis and hypothalamus to vital dyes, with a study of the hypophyseal vascular supply." Amer.J.Anat. 58, 421-472.

WISLOCKI,G.B. & LEDUC,E.H. (1952) "Vital staining of the hematoencephalic barrier by silver nitrate and trypan blue, and cytological comparisons of the neurohypophysis, pineal body, area postrema, intercolumnar tubercle and supraoptic crest." J.comp. Neurol. 96, 371-414.

WOODS,L.A., COCHIN,J., FORNEFELD,E.J., McMAHON,F.G. & SEEVERS,M.H. (1951) "The estimation of anions in biological materials with critical data for cocaine and mescaline." J.Pharmacol. 101, 188-199.

WOODS,L.A., McMAHON,F.G. & SEEVERS,M.H. (1951). "Distribution and metabolism of cocaine in the dog and the rabbit." J.Pharmacol. 101, 200-204.

WOODWORTH,S. (1903). "The electric conductivity of mammalian nerve." Thompson Yates and Johnston Lab. Rep. 5, part 1, 61-66.

WRIGHT,E.A., MORGAN,R.S. & WRIGHT,G.P. (1950). "Tetanus intoxication of brainstem in rabbits." J.Path.Bact. 62, 569-583.

YUIEN,K. (1928). "On the direction of the lymphatic stream in the nerve." Folia,anat.Jap. 6, 301-309.

YUIEN,K. & SATO,K. (1929). "On the spreading path of stains injected into the nerve." Folia anat. Jap. 7, 419-423.

ZWEIFACH,B.W. (1937). "The structure and reactions of the small blood vessels in amphibia." Amer. J.Anat. 60, 473-514.

DAILEY,M.E. (1931). "The equilibrium between cerebrospinal fluid and blood plasma. 6. The distribution of sodium between cerebrospinal fluid and blood serum". J.Biol.Chem. 93, 5-15.

FREMONT-SMITH,F., DAILEY,E.M., MERRITT,H.H., CARROLL, M.P. & THOMAS,G.W.(1931). "The equilibrium between cerebrospinal fluid and blood plasma. 1. The composition of the human cerebrospinal fluid and blood plasma". Arch.Neurol.Psych. 25, 1271-1289.

FREMONT-SMITH,F., DAILEY,M.E., MERRITT,H.H., & CARROLL,

M.P., (1931 b). "The equilibrium between cerebrospinal fluid and blood plasma. 2. The composition of the human cerebrospinal fluid and blood plasma in meningitis". Arch. Neurol.Psych. 25, 1290-1296.

ERLANGER,J. and BLAIR,E.A. (1934) "Manifestations in myelinated axons." Amer.J.Physiol. 110, 287-311.